The refinement of protective salinity guidelines for South African freshwater resources.

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Abstract

South Africa is an arid country and its growing population is putting freshwater resources under increasing pressure. Natural salinization of freshwater systems is being exacerbated by anthropogenic influences. The National Water Act (No. 36 of 1998) stipulates the need for an ecological Reserve, that quantity and quality of freshwater needed to protect freshwater ecosystems while allowing sustainable use of freshwater resources. Water guidelines do exist in the form of the South African Water Quality Guidelines (DWAF, 1996) and more recently, Jooste and Rossouw (2002) compiled benchmark values for water quality variables marking the boundaries between ecological health classes in the 4-category classification system. Predominantly international toxicity data were used to compile the guidelines and the benchmark values. In addition, there is a paucity of chronic toxicity data nationally and internationally. This thesis showed that it is statistically possible to derive protective chronic endpoints for salinity from acute toxicity data through extrapolation. The Acute to Chronic Ratio (ACR), Two-Step Linear Regression (LRA) and Multi-Factor Probit Analysis (MPA) extrapolation methods were investigated to derive chronic toxicity data from acute toxicity data. The authors of LRA and MPA recommend associating a time independent LC_x value in the range of $LC_{0,01}$ to LC_{10} with a Predicted No Effect Concentration (PNOEC). In addition to published methods, this thesis studied the possibility of equating a time independent LC_{50} value and subjected to a safety factor of 5 (LRA $LC_{50}/5$), to the PNOEC. Extrapolated chronic toxicity data where the toxicants are NaCl and Na_2SO_4 were derived for indigenous South African macroinvertebrates. NaCl and Na_2SO_4 are salts associated with salinisation in South Africa. In addition, a chronic salinity toxicity test protocol for an indigenous South African aquatic macroinvertebrate was designed and chronic toxicity test were performed using NaCl and Na_2SO_4 as toxicants. The experimental chronic toxicity data produced were used to validate results from the acute to chronic extrapolation methods. Extrapolated chronic toxicity data were inputted into Species Sensitivity Distribution curves, and concentrations that were predicted to protect 95 % of species (PC95) were compared to the sub-lethality benchmarks proposed by Jooste and Rossouw (2002) for NaCl and Na_2SO_4. This study concluded that the LRA $LC_{50}/5$ extrapolation method is the most protective and

accurate and proposed that LRA replace the ACR method in future guideline development for inorganic salts.

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Declaration

The following thesis has not been submitted to a university other than Rhodes University, Grahamstown, South Africa. The work presented here is that of the author.

Chapter 1: General Introduction

Water is a basic requirement for life. People need water for domestic, agricultural, industrial and recreational use. Freshwater is however limited, with only 1,7 % of the Earth's total water available as groundwater and 0,014 % of the Earth's total water available as surface water (Sorooshian *et al*., 2002). Water is, however, taken for granted as huge volumes of water are often used without much thought of the consequences. For instance, it is estimated that one kilogram of grain grown in an irrigated arid area uses approximately 1 ½ - 2 ½ m^3 of water (Smedema and Shiati, 2002). In addition, human activities are polluting freshwater systems.

Water resources in South Africa are almost fully utilised and most are under stress as a result of over- abstraction, flow regulation and pollution due to population growth, increased economic activity and changes in land use (Walmsley *et al*., 1999). It is estimated that demand for water in South Africa will double in the next 30 years and water is, and will be the limiting factor to development in South Africa (O'Keeffe, 1989; Walmsley *et al*., 1999).

1.1 South Africa's climate and rainfall

Climate and rainfall in South Africa is variable for different regions and this affects the degree of freshwater salinisation in these different regions. South Africa is an arid country and evaporation exceeds rainfall in most parts of the country (Day, 1993). Rainfall exceeds evaporation only in the north - east and south - west of the country with Lake Fundudzi being the only permanent freshwater lake in South Africa and only approximately 9 % of rainfall appearing as river flow (Day, 1993). Rainfall in South Africa is below world average and only exceeds 2500 mm/year in the south - western Cape and Kwazulu – Natal (DWAF, 1996). Precipitation averages 497 mm per annum compared to the international average of 860 mm (Walmsley *et al*., 1999).

The warm Agulhas current occurring off the east coast and the cold Benguela current occurring off the west coast strongly influence the climate and temporal and spatial

variability in climatic conditions across the country have been the motivation to define five different climatic regions in southern Africa (Walmsley *et al*., 1999). Region one encompasses the Northern Province and northern Kwazulu - Natal bordering with Mozambique. The region is defined as a marine influenced coastal belt with a sub – tropical climate and rainfall. Region two encompasses the Gauteng region, North West Province and the Free State in the north stretching to the south - eastern coastal plain between East London and Durban. This region experiences summer rainfall and includes the Vaal - Orange, Thukela and Limpopo rivers. The mountains of Lesotho form their own region, with high rainfall. A large region of South Africa stretching from Port Elizabeth in the south, northwards into the Northern Cape is defined as arid. The western part of this region is dry with temporary waters. The eastern coastal section near Port Elizabeth is characterised by short rivers with large gradients and permanent flows. The southern regions of the Eastern Cape and the Western Cape has a Mediterranean climate with winter rainfall.

1.2 Natural salinization of surface waters

Natural salinization occurs as a result of the weathering of rock by primarily physical but also chemical and biological processes (Pillsbury, 1981). The mobilization of fossil salt of the substrata of a catchment and atmospheric precipitation are also factors that contribute to natural salinization Day (1993). During precipitation, water carries salts and rock particles from highland to lowland areas (Pillsbury, 1981). In addition, rainwater causes a mobilisation of fossil salts through a process called leeching (Smedema and Shiati, 2002). These salts are carried into rivers by rainwater. Other causes of natural salinization include the deposition of airborne oceanic aerosols via rainfall and the process of evaporation concentrates dissolved salts in surface waters (Anon, 1990; Herczeg *et al*., 2001). Salinization is mostly restricted to arid regions of the world (25 – 500 mm rain per annum), such as large parts of southern Africa, Central and South America, some parts of North America, the Middle East, Central Asia and parts of Australia (Williams, 2001). Salinization occurs in both surface and ground water (Herczeg *et al*., 2001).

The concentration of salt and dominant salts occurring in terrestrial water bodies is dependent on geographical location. Day (1993) and Day and King (1995) found that

geographical patterns of ionic dominance occur in the rivers of South Africa and have classified inland water systems of southern Africa according to major ion chemistry. The salt sodium chloride dominates inland waters while calcium carbonate is dominant in the dilute waters of Gauteng and Kwazulu - Natal. Day (1993) correlates sodium chloride with inland water bodies subject to high degree of evaporation. Sulphates form a small proportion of the salts found naturally in inland waters. Day and King (1995) found that the extreme south western parts of the country are dominated by sodium and chloride ions while less than 100 km inland and along the south eastern coast, different proportions of calcium, magnesium, sodium, hydrogen carbonate, sulphate and chloride ions dominate. The salts calcium carbonate and calcium sulphate are the first salts to precipitate out as salt concentrations increase due to evaporation, leaving only sodium and chloride in solution. Water resources in the north - western regions of the Northern Cape tend to be temporary and usually saline (Walmsley *et al*., 1999). The mountain regions of Lesotho, the Nothern Cape, Kwazulu - Natal and eastern Gauteng contain mountain streams with low salinity (Day and King, 1995; Walmsley *et al*., 1999). Rivers in the south - eastern coastal belt near Port Elizabeth are characterised by moderate salinity levels (Walmsley *et al*., 1999). Salinity levels of aquatic ecosystems in the eastern Gauteng and northern Kwazulu - Natal are variable (Walmsley *et al*., 1999).

1.3 The problem of freshwater salinization due to anthropogenic factors

Anthropogenic modification of aquatic ecosystems has been happening in many countries at a rapid pace over the last 100 years (Masteller, 1993). For example, water consumption has reduced the Colorado and Rio Grande rivers in the USA, the Yellow River in China, the Euphrates River in Iraq and the Murray River in Australia from once mighty flowing rivers to insignificant streams (Smedema and Shiati, 2002). Globally, extinctions of aquatic flora and fauna may be as much as 3000 species per year (Ormerod, 1999). In the future, many arid countries will most likely experience shortages of fresh water because of fast growing populations (Williams, 2001).

A large portion of southern Africa has been identified as a currently severely water stressed area (Alcamo and Henrichs, 2002) and aquatic ecosystems in South Africa are generally in an altered state (Walmsley *et al*., 1999). Increasing demand for water

has led to all major rivers in South Africa being regulated (Walmsley *et al*., 1999). Surface water is becoming polluted by industrial effluents, sewerage, irrigation return flow, litter and acid mine drainage and many rivers are experiencing increasing nutrient and salinity levels (Ormerod, 1999; Walmsley *et al*., 1999). Catchment degradation, over extraction of water, habitat loss and the breakdown of biogeographical barriers due to inter-basin transfers are other problems impacting on the state of aquatic ecosystems in South Africa (Walmsley *et al*., 1999). Human induced ecological changes to aquatic ecosystems in South Africa have resulted in a loss of biodiversity and an increase in certain invasive or pest species (Walmsley *et al*., 1999). As a consequence, many aquatic faunal and floral species are in danger of extinction in South Africa (Walmsley *et al*., 1999).

A Water Research Commission workshop held in 1989 recognized that there is a strong trend of increasing salinity in South African water resources concealed by variable rainfall and other factors and identified the following main causes (Anon, 1990):

- Flow reduction in rivers.
- Evaporative losses in surface waters.
- Irrigation return flow.
- Runoff from dry-land agriculture.
- Saline industrial effluents.
- Urban development contribution point and non-point sources.
- Atmospheric deposition.

Salinization of freshwater resources has a direct economic cost as water with a salinity of higher than 1000 mg/L becomes useless for agriculture, human consumption and most industrial purposes (Williams, 2001).

The indigenous aquatic fauna and flora of southern Africa are typically adapted to temporal and spatial variability in physical conditions and many are opportunistically reproductive (Walmsley *et al*., 1999). Anthropogenic influence often breaks down this natural variability, for example seasonally flowing rivers becoming perennial and vice

versa (Walmsley *et al*., 1999). Salinity levels in many impacted rivers are on average higher than the average natural levels with less temporal variability (Kefford *et al*., 2002). Salinization has in the past been held in check by seasonal floods that have been effective in carrying salts to the oceans (Pillsbury, 1981). Flow regulation and high consumption of released water has disturbed this natural balance and salts are being retained within river basins (Pillsbury, 1981). Under natural conditions, periodic salinity fluctuations probably structured aquatic communities. Higher mean salinities in modern times have probably excluded the possibility of refuges for aquatic communities during high salinity periods, which in turn has decreased the level of re-colonisation of rivers once salinities decrease (Kefford *et al.*, 2002). Although rivers are typically resilient and recover quickly from short - term perturbations, many studies have shown that rivers are not resilient to sustained disturbance as even refugia become disturbed (O'Keeffe, 2000). Higher mean salinities could well be classified as a sustained disturbance.

Salinization of rivers is occurring globally. Williams (2001) cites salinization as occurring in the Colorado River in North America, the Syr-Darya and Amu-Darya rivers in Central Asia and the Blackwood River in Australia where salinity in the upper reaches has increased twenty fold from historical levels. The headwaters of the Colorado River are of good quality with low salinity levels approximating 50 ppm. Return flow from agriculture has however contributed to higher salinity levels approximating 800 ppm by the time the Colorado River reaches the Mexican border (Sorooshian *et al*., 2002). In Australia salinization is a natural process that happens during drought periods (Kefford *et al*., 2002). The replacement of natural deep - rooted vegetation in river catchments with shallow - rooted agricultural vegetation and salt discharge from mining are however contributing to higher salinity levels in freshwater resources (Williams 2001). In addition, river regulation in Australia has resulted in higher mean salinities (Kefford *et al*., 2002).

Irrigation in arid regions has been identified as a major contributor to freshwater salinization (Williams, 2001). Irrigation in arid regions such as South Africa leads to an accumulation of salts in agricultural land and water resources (Smedema and Shiati, 2002). Extensively vegetated agricultural land in arid regions has a high evapo-transpiring surface. Therefore salts found in water used to irrigate crops in these areas

become concentrated in the land (Smedema and Shiati, 2002). It is estimated that up to five tons of salt is concentrated per irrigated hectare per year in arid regions (Smedema and Shiati, 2002). Return flows from irrigated agricultural land transport these salts as well as primary fossil salts from the underlying substrata into water resources (Smedema and Shiati, 2002). Land salinization has become a problem for crop farmers due to irrigation. However, solving this problem with better saline water drainage will lead to more saline water entering water freshwater resources (Smedema and Shiati, 2002) unless drainage water is treated.

The effects of salinity differ for different components of an aquatic ecosystem. High salinity may cause suspended sediments to precipitate, thereby making the water clearer (WRC, 2000). This would have a direct effect on algal productivity. Aquatic fauna and flora are hyper-osmotic regulators. Since plants and animals contain more ions than the surrounding medium, they are constantly excreting dilute urine and taking up ions. High salinity levels will disrupt this process and cause a drop in productivity (WRC, 2000). High salinities may also reduce the growth of riparian vegetation causing river bank instability (WRC, 2000).

Various studies have highlighted the problem of fresh water salinization to aquatic ecosystems. Kefford and Doeg (1999) found a general decline in abundance and species richness of macro-invertebrate fauna in irrigation canals in the Shepparton irrigation region of Australia, which was attributed to saline ground water disposal. Kefford (1999) found a general decrease in abundance of macro-invertebrates associated with saline water disposal from Sanctuary Lake into the Barwon River in Australia. Kefford and Robley (1996) investigated the effect of saline water disposal in the Barwon River and Birregurra Creek in Australia. They found that saline water disposal decreased the abundance of macro-invertebrate taxa and altered the macro-invertebrate community structure in Birregurra Creek and in the Barwon River downstream of Birregurra Creek. Blinn and Bailey (2001) aimed to identify diatom indicator species and assemblages along gradients of secondary (anthropogenic) salinization in lowland streams throughout Victoria in Australia. They showed that lowland streams in areas with high anthropogenic salinization showed lower diatom species diversity. Although Kefford (1999) has highlighted the negative effect of salinity increases in natural systems, he has also stressed that salinity is working in

combination with other stressors and the effect of salinity itself in many cases cannot be isolated. Species richness usually decreases in aquatic ecosystems subjected to elevated salinity levels. A popular theory in conservation ecology is that the maintenance of natural biodiversity is the key to the health of ecosystems and their sustainable utilization (O'Keeffe, 2000; WRC, 2000). A definition of biodiversity would be appropriate at this stage. For convenience, I am using the Tilman (1997) definition of biodiversity, i.e. biodiversity meaning the number of species in an ecosystem. Naeem *et al.* (1994) proved that declining biodiversity alters the performance of ecosystems by demonstrating that higher diversity plant communities consume more carbon dioxide than lower diversity communities and therefore showed higher productivity. Tilman and Downing (1994) showed that higher diversity grassland were more resistance to perturbation and more resilient than lower diversity grassland. The maintenance of natural biodiversity may mean that some species are functionally redundant i.e. more than one species in a particular ecosystem may perform the same ecological role. Functional redundancy may play a role in maintaining an ecosystem's resistance to disturbance and an ecosystems degree of resilience as functional redundancy means that there is another species available to fill the ecological role of a compromised species within an ecosystem (De Leo and Levin, 1997; Cairns, 1983). The degree of functional redundancy may be linked to the resilience of an ecosystem, as the ecological role performed by a compromised species may be 'taken over' by another functionally redundant species (De Leo and Levin, 1997). The diversity – stability hypothesis states that ecosystems with healthy biodiversity are more likely to contain some species that can survive and perform essential ecological functions during a disturbance (Tilman and Downing, 1994). Tilman and Downing's (1994) research with grassland supported the diversity – stability hypothesis. Besides only maintaining the health of ecosystems, biodiversity should be maintained because many species have economic and aesthetic value (De Leo and Levin, 1997). In reality, the relationship between biodiversity and ecosystem function is not well understood, and this is a reason in itself to preserve biodiversity as a precautionary measure (De Leo and Levin, 1997).

1.4 The ecological Reserve

South Africa's freshwater resources are utilized by the industrial, agricultural and domestic sectors of South Africa and are subject to intensive use. Water is likely to be the limiting factor to development in South Africa in the future and it is important to develop a culture of sustainable water use (Walmsley *et al*., 1999). The persistence of some diffuse pollutants indicates that it is wiser to protect aquatic ecosystems from degradation than to plan to implement recovery procedures after degradation has taken place (Ormerod, 1999; O'Connor *et al*., 2003). Anthropogenic disturbance of aquatic ecosystems, if left until disaster levels, are often complex and widespread making the costs of rehabilitation prohibitive (O'Connor *et al*., 2003). South Africa and other countries in Africa, stand at a critical crossroad in terms of aquatic ecosystem protection, in that aquatic ecosystems in Africa have a high aquatic biodiversity but little is known about how to protect them (Ormerod, 1999). There is therefore the need for legislation to ensure freshwater resources in South Africa are used in a sustainable manner and protected in the long term.

The South African National Water Act (No. 36 of 1998) stipulates the requirement for an ecological Reserve. The National Water Act defines the ecological Reserve as the water required in terms of both quantity and quality, to sustain the health of aquatic ecosystems while allowing sustainable use of water resources. The ecological Reserve was not put in place solely for the benefit of aquatic ecosystems. Ultimately healthy aquatic ecosystems provide goods and services for mankind, for example natural water purification systems, food sources such as fish and recreation possibilities amongst other benefits (ANZECC, 1992; DWAF, 1996; Palmer *et al*., 2002). Since salinization is a water quality problem, the ecological Reserve needs to be specified in terms of salinity levels that are not harmful to aquatic ecosystems but still allow economic development.

1.5 Water quality guidelines

The water quality aspect of the ecological Reserve can be quantified in terms of water quality guidelines. Water quality guidelines are numerical concentration units or narrative statements for substances recommended to support and maintain a

designated water use or ecosystem condition (ANZECC, 1992). Results of aquatic ecotoxicology tests form a valuable resource for the compilation of water quality guidelines as aquatic toxicology attempts to define a cause – effect relationship between aquatic organisms and a particular toxicant (Rand, 1995). Aquatic toxicology tests are broadly classified as either acute or chonic (Rand *et al*., 1995). Acute toxicity tests are typically of short duration and usually measure lethality (Rand *et al*., 1995). Chronic toxicity tests are usually run over a test organism's entire lifecycle or at least a large proportion of the lifecycle and sub-lethal endpoints such as reproduction or growth are usually measured (Rand *et al*., 1995). The definition of chronic toxicity data used by the ANZECC and ARMCANZ (2000) guidelines and Jooste and Rossouw's (2002) benchmarks differ. While the ANZECC and ARMCANZ (2000) guidelines consider all toxicity tests for multicellular organisms of an exposure duration of greater than 96 hours as chronic, Jooste and Rossouw (2002) have taken a different approach to distinguishing chronic toxicity data, with toxicity data measuring sub-lethal biological responses being considered as chronic. This thesis has adopted the ANZECC and ARMCANZ (2000) definition of chronic toxicity data, as long - term survival of tests organisms to a toxicant is a valid biological measure within chronic toxicity tests and the inclusion of survival as a valid chronic biological measure greatly increases the amount of usable existing chronic toxicity data.

Various countries have compiled their own sets of water quality guidelines, for example the USA (USEPA, 1986), Canada (CCREM, 1991), Australia (ANZECC and ARMCANZ, 2000) and South Africa (DWAF, 1996). A short review of water quality guidelines used in South Africa and Australian guidelines follows here. The Australian and New Zealand Water Quality Guidelines (ANZECC and ARMCANZ, 2000) are argumentatively considered the most relevant guidelines to discuss in addition to those used in South Africa as the Australian and New Zealand Water Quality Guidelines (ANZECC and ARMCANZ, 2000) are arguably the most environmentally realistic water quality guidelines produced up to date because of the sophisticated methods used, although the use of sophisticated methods do not guarantee environmental realism.

1.5.1 The South African Water Quality Guidelines

The South African Water Quality Guidelines (DWAF, 1996) were developed by the Department of Water Affairs and Forestry (DWAF), with the aim of improving decision support tools required for the management of water resources. Volume 7 of the guidelines are specifications of surface water quality required to protect freshwater aquatic ecosystems. The guidelines (DWAF, 1996) provide a set of information for a specific water quality constituent in terms of a Target Water Quality Range (TWQR), a Chronic Effect Value (CEV) and an Acute Effect Value (AEV) together with support information and guidelines for site – specific modifications to water quality information (DWAF, 1996). The TWQR specifies the perceived safe concentration range of a water quality constituent for a particular resource to protect the aquatic ecosystem from continuous exposure (DWAF, 1996). The CEV is used where the TWQR is exceeded and the AEV identifies cases requiring urgent management. The CEV is a level of toxicant that is expected to cause significant chronic effects to 5 % of the species in an aquatic community, so theoretically, any concentration below the CEV should protect at least 95 % of species from long-term exposure. The AEV is the concentration of a toxicant that is expected to have acute effects on 5 % of species. The South African Water Quality Guidelines (DWAF, 1996) relied on a method developed by the USEPA to derive the CEV and AEV values. The method relies on toxicity data for a representative range of organisms. Since local toxicity data is lacking, the South African Water Quality Guidelines (DWAF, 1996) relied predominately on international toxicity data contained on the ASTER and AQUIRE toxicity databases (Roux *et al.*, 1996). Roux *et al*. (1996) gives a good explanation of the calculation procedure used. Chronic toxicity data (as the geometric mean of the LOEC and NOEC) were grouped into taxonomic means according to test organisms, with toxicity results being grouped into species and genus means. Final chronic values were calculated by fitting genus means to a log triangular distribution. The Final Chronic Value (FCV) was taken as the 5^{th} percentile on the distribution, i.e. a concentration that theoretically should protect 95 % of species from chronic effects. Depending on the range of taxonomic groups represented by the chronic toxicity test organisms, the Final Chronic Value was subjected to a safety factor to derive the Chronic Effect Value (CEV). A similar procedure was used to derive the AEV, except acute toxicity data were used. Where there were not enough chronic toxicity data

available to derive a CEV, the Final Acute Value (FAV) was subjected to an Acute to Chronic Ratio (ACR).

1.5.2 Ecological Reserve boundary values

Jooste and Rossouw's (2002) proposed generic benchmarks were aimed at quantifying the water quality ecological Reserve required under the National Water Act (no 36 of 1998). Jooste and Rossouw (2002) aimed to derive generic class-related water quality management benchmarks and suggested that river ecosystem health in South Africa should be classified according to the following classes:

- Excellent.
- Good.
- Fair.
- Poor.

In the benchmarks, potential hazard to aquatic ecosystems are equated to loss of species. Similar to the procedure used in the South African Water Quality Guidelines (DWAF, 1996), generic benchmarks are adapted to the needs of particular aquatic ecosystems i.e. become site specific. Toxicological databases, such as the USEPA ECOTOX database were treated as sources of generic stressor-response information and were used to derive the lethality and sub-lethal effects generic benchmarks for particular stressors, where 95 % protection from lethal effects and sub-lethal effects were aimed for in each benchmark respectively. Within a Generic Stressor Response Relationship (GSSR) the sub-lethal effects benchmark was associated with the boundary between the Good and Excellent ecological health classes, effectively marking the upper limit of the no-hazard domain. The lethality benchmark marked the upper limit of the transitional hazard domain and defined the boundary between the Fair and Poor ecological health classes. Jooste and Rossouw (2002) distinguished between toxicological data for the sub-lethal effects and lethality benchmarks by the biological responses measured in the toxicity tests and not the test durations. Toxicity tests that measured sub-lethal biological responses (e.g. fecundity and growth) were used to define the sub-lethal effects benchmark while toxicity tests that measured lethality were used to define the lethality benchmark (Jooste and Rossouw, 2002).

1.5.3 Use of toxicological measures to define site – specific class related boundary values

Scherman *et al*. (2003) attempted to link ecotoxicology, water chemistry and biomonitoring so as to integrate water quality into environmental flow assessments. The process was developed during environmental flow assessments and ecological reserve studies undertaken for the Olifants, Breede and Thukela river catchments in South Africa. Results of 96 hour acute and 10 day short – term chronic toxicity tests were used to set boundaries between ecological management classes for the rivers assessed. Lower 95 % confidence limits of LC_1s determined from regression analysis of short – term chronic tests were used to set the boundary between the Excellent and Good management classes. The measured LC_1 or LC_5 of short – term chronic tests were used to set boundaries between Good and Fair management classes. LC_1s determined from acute toxicity tests were used to set the boundary between the Fair and Poor management classes.

1.5.4 The ANZECC and ARMCANZ (2000) approach to developing water quality guidelines

The ANZECC and ARMCANZ (2000) guidelines are more appropriately termed trigger values as exceedance of the trigger values prompts or 'triggers' further management action (Warne, 2001). The process of deriving the ANZECC and ARMCANZ (2000) trigger values used both a statistical species sensitivity distribution method and an assessment factor method (Warne, 2001). These were the recommendations of a review of guideline derivation methods by Warne (1998). According to available tolerance data, trigger values were divided into high reliability (HR), medium reliability (MR) and low reliability (LR) categories (Warne, 2001). Mesocosm, single species chronic and single species acute toxicity data were collected from international ecotoxicity databases such as AQUIRE as well as published data (Warne, 2001). The process of developing the ANZECC and ARMCANZ (2000) trigger values took a fundamentally different approach to distinguishing between toxicological data than was used by Jooste and Rossouw (2002). Toxicological data were distinguished by exposure duration into either the acute or chronic category, and not on lethality or sub-lethal biological responses

measured in the tests. The process of deriving the ANZECC and ARMCANZ (2000) trigger values first tried to derive high reliability (HR) trigger values (TVs) using mesocosm or single species chronic toxicity data using the Species Sensitivity Distribution (SSD) method or the Application Factor (AF) method (Warne, 2001). Certain rules over the number of taxonomic groups and species represented in the tolerance data were in place for the use of the SSD method. SSDs utilise all available tolerance data and not just the most sensitive toxicity data obtained from usually robust laboratory reared species (Warne, 1998). The value of the application factor used in the AF method also depended on the available tolerance data, with the application factor value being inversely proportional to the quantity and quality of available tolerance data (Warne, 2001). If insufficient tolerance data were available to derive HR TVs, medium reliability (MR) TVs were attempted using predicted chronic data derived by the application of Acute to Chronic Ratios (ACRs) to acute data using the SSD and AF methods (Warne, 2001). Low reliability (LR) TVs were derived by using either estimates of chronic toxicity data derived by Quantity Structure – Activity Relationships QSARs in an SSD, or the AF method (Warne, 2001). QSARs are simple models that relate the biological activity of chemicals to physico- chemical properties or molecular descriptions of the chemicals (Warne, 1998) and were restricted to non-polar narcotic toxicants in the derivation of the ANZECC and ARMCANZ (2000) trigger values (Warne, 2001). Warne's (1998) review of guideline derivation methods recommended the Aldenburg and Slob (1993) SSD method. This method assumes that species sensitivity follows a log-logistic distribution (Warne, 2001). There is no scientific reason to assume this but the distribution allows the use of convenient mathematical features (Warne, 2001 citing Aldenburg and Slob, 1993). Shao (2000 cited in Warne, 2001) developed a family of species sensitivity distributions called the Burr Type III (BT III) in which the log-logistic distribution was included. As this method is likely to include a model that fits any given species sensitivity distribution, it was adopted for the ANZECC and ARMCANZ (2000) trigger values (Warne, 2001). The SSD method produces a concentration value of a toxicant that should protect x percent of species called a PC_x value (Warne, 2001). Associated with the PC_x value is a confidence limit that indicates the degree of certainty that the PC_x value will protect x % of species. This is needed, as only a sample of the species in the environment is represented in any particular SSD, and depending on the species represented, an SSD for any particular chemical will

produce different TVs (Warne, 2001). The ANZECC and ARMCANZ (2000) trigger values use a standard of 50% for this confidence index, as it is the most statistically robust (Fox, 1999 cited by Warne, 2001). The value of the application factor used by the AF method in the derivation of the ANZECC and ARMCANZ (2000) trigger values was governed by three extrapolations, i.e. laboratory to field, acute to chronic and few to many species (Warne, 2001). Each extrapolation had a value of 10 and the final application factor was the product of all extrapolation values (Warne, 2001), so for example, a MR TV application factor was the product of a laboratory to field extrapolation of 10, and an acute to chronic extrapolation of 10 to give a value of 100. The 'few to many species' extrapolation was used when there were few species represented in the available tolerance data. ACRs only defaulted to 10 where an ACR could not be calculated by available acute and chronic tolerance data (Warne, 2001).

1.6 Protective salinity guidelines

1.6.1 Salinity guidelines within the South African Water Quality Guidelines (DWAF, 1996)

The South African Water Quality Guidelines (DWAF, 1996) treat Total Dissolved Salts (TDS) as a non-toxic inorganic constituent (DWAF, 1996). TDS was defined as all dissolved compounds within water that carry an electrical charge (DWAF, 1996). The evaluation of TDS described in the guidelines in the form of a TWQR, included the consideration of the changes from local natural cycles. The TWQR specified that TDS concentrations should not be changed by greater than 15% from the normal cycles of the water body under un-impacted conditions at any time, and that natural seasonal fluctuations in TDS should be maintained (DWAF, 1996). The South African Water Quality Guidelines (DWAF, 1996) also offer guidelines for specific ions of which some are major contributors to salinization. These ions are classified as toxic constituents and therefore the numerical AEV and CEV values are used to specify safe levels.

1.6.2 Ecological Reserve boundary values for salts associated with salinisation

Jooste and Rossouw's (2002) benchmarks consider individual salts, and do not aggregate salts into a TDS measure. Their method uses the Toxicologically Important

Major Salts (TIMS) model, which calculates salt combinations from ionic concentrations in a water sample (Jooste and Rossouw, 2002). The method puts ions into a 'salt bank', where ions are attributed to the most toxic possible salts first, with less toxic salts getting lower 'bidding rights' to the ions. Although the TIMS model is a conservative measure in that toxicants in a mixture often act to inhibit each other, the possibility that certain salts may have a supra-additive effect when mixed should not be discounted. In this case, the TIMS model would underestimate toxicity.

1.6.3 Site – specific, class related boundary values for salinity obtained from toxicological measures

Scherman *et al*. (2003) attempts to integrate water quality into environmental flow assessments and identified salinization as the driving water quality issue in the Olifants, Breede and Thukela river catchments. The toxicity test process involved toxicity testing on site specific macroinvertebrates using the salts commonly associated with salinity i.e. sodium chloride and sodium sulphate.

1.7 Problems with current protective salinity guidelines

A Water Research Commission workshop held in 1989 identified the need for supplementary research to be done before applying international research to the South African situation (Anon, 1990). Both the South African Water Quality Guidelines (DWAF, 1996) and Jooste and Rossouw's (2002) boundary values fail in this respect. The South African Water Quality Guidelines (DWAF, 1996) relied upon the results of toxicity tests on a representative range of aquatic organisms. However, the data were sourced primarily from the United States Environmental Protection Agency (USEPA, 1986) ASTER and AQUIRE databases, the Canadian Water Quality Guidelines (CCREM, 1987) and the Australian Water Quality Guidelines (ANZECC, 1992), and therefore little locally derived data were used (Roux *et al*., 1996). Jooste and Rossouw (2002) also used predominantly international toxicity data sources mainly from the USEPA toxicity databases. Therefore the boundary values given by Jooste and Rossouw (2002) and the guidelines (DWAF, 1996) both give predictions of responses for organisms that do not exist naturally in the resources to be protected. South African aquatic biota are not likely to differ dramatically in salinity tolerance compared to biota from other part of the world (Palmer *pers.comm.*, 2004). However

certain taxa that are tolerant of high salinity may dominate South African freshwater systems and it is therefore important to locate the levels of tolerance of South African taxa in the context of international data. In addition, salinity tolerance information for indigenous biota will provide for accurate site-specific assessments (Palmer *pers.comm.*, 2004).

1.8 Aims

Chronic salinity tolerance data, compared to acute toxicity data, are much harder to obtain because of the relative expense and difficulty involved. This is reflected in the lack of chronic salt tolerance data available both nationally and internationally. Ion specific salinity guidelines given by the South African Water Quality Guidelines (DWAF, 1996) and benchmark boundary values proposed by Jooste and Rossouw (2002) need to be validated, and amended if necessary, by the results of chronic salinity toxicity data for aquatic biota indigenous to South Africa. Therefore there is a need for both chronic experimental research and reliable extrapolation methods to convert acute response data to chronic response data. Sodium chloride and sodium sulphate have been identified as major salts involved in salinization of aquatic ecosystems in South Africa. Sodium chloride (NaCl) is linked to natural and agriculturally caused salinization while sodium sulphate (Na_2SO_4) is linked to mining and industrial caused salinization (Scherman *et al*,. 2003). The aims of this thesis are therefore:

1. Chapter 2 will explore the derivation of statistically valid chronic endpoints from acute toxicity data.
2. Chapter 3 provides a comprehensive set of chronic data with sublethal endpoints for an indigenous aquatic macroinvertebrate against which results of acute to chronic extrapolations can be benchmarked.
3. Chapter 4 uses experimentally measured acute and chronic responses to assess the extrapolation methods explored in Aim 1.

This thesis also discusses the potential role of extrapolated chronic data in protective guideline development.

Chapter 2: Acute to chronic extrapolation in the estimation of the chronic salinity tolerance of aquatic macroinvertebrates.

2.1 Introduction

Aquatic ecotoxicity data are a valuable resource for the compilation of water quality guidelines. There are few chronic toxicity data available for aquatic organisms compared to the relative abundance of acute toxicity data. Yet, chronic toxicity data are widely considered to be more protective than acute toxicity data. In fact, the Australian and New Zealand Water Quality Guidelines (ANZECC and ARMCANZ, 2000) recognise that acute data cannot provide sufficient protective guidelines for aquatic ecosystems, and chronic data are estimated from acute data by the application of acute to chronic ratios (ACRs) or safety factors (Warne, 2001). Chronic toxicity testing is generally more difficult to perform and requires more resources in terms of time and money. Acute to chronic data extrapolation techniques could therefore be invaluable provided an accurate extrapolation technique is found.

At this point, definitions of acute and chronic toxicity tests and their respective toxicity measures would be useful, as these terms are used extensively in this chapter. Rand (1995) defines acute toxicity tests as experiments that generally run for four days or less and where mortality is usually the response measured. Chronic toxicity tests are defined as tests that last for a longer time than acute tests, depending on the reproductive cycle of the test animal (Rand, 1995). Sub-lethal endpoints such as those associated with reproduction and growth in addition to survival are generally measured in chronic toxicity tests. Two toxicological measures are commonly used to express tolerance although many others are in existence. Both measures are expressed as a concentration unit of the toxicant. The Median Lethal Concentration (LC_{50}) is a time dependant variable and expresses the concentration of a toxicant that is predicted to cause death to 50 % of the test organisms at a given exposure period (Rand, 1995).

LC_{50} measures are most often associated with acute tests, but can be the endpoint of short-term chronic tests (Scherman *et al*., 2003) and are determined by regression analysis (Rand, 1995). The No Observed Effect Concentration (NOEC) is determined by Analysis of Variance statistical techniques and is the highest concentration within a toxicity test concentration range that does not have a significant adverse effect on the test organisms when compared to the control (Rand, 1995). The NOEC is usually associated with chronic toxicity tests.

Lee *et al*. (1995), Mayer *et al*. (1994) and Mount and Stephan (1967) proposed methods of extrapolating chronic toxicity data from acute toxicity data. These methods can all broadly be classified as either assessment factor methods or statistical methods (Warne, 1998).

2.1.1 Assessment factor method for extrapolating acute to chronic data

2.1.1.1 The Acute to Chronic Ratio (ACR)

The Acute to Chronic Ratio (ACR) is classified as an assessment factor method (Warne, 1998). An Acute to Chronic Ratio (also called an Acute to Chronic Toxicity Ratio) is a unit less numerical value that should be greater than 1, obtained by dividing an acute measure for a particular organism for a particular toxicant by a chronic measure for the same toxicant and organism (Rand, 1995; Warne, 1998). Importantly, the responses measured in the respective acute and chronic measures do not have to be the same, e.g. mortality for one and fecundity for the other. This ratio is often used to estimate possible chronic toxicity based on the acute toxicity of a substance where some chronic toxicity data for an organism exists (Rand, 1995). ACRs are usually used as a last resort to generate chronic toxicity data when there is little information available (Giedy and Graney, 1989). Warne (1998) lists the use of two types of ACR, namely a generic ACR and a chemical specific ACR. Generic ACRs are not chemical specific and typical values include 2 (CCREM, 1991) or 10 (CCREM, 1991; USEPA, 1986). Mount and Stephan (1967) first proposed the use of chemical specific ACRs for fish exposed to Malathion and Butoxy-ethonol ester of 2,4-D. Warne (1998) lists the ACRs as generic, but the authors clearly derived an ACR for each chemical, namely 45 and 19 for Malathion and Butoxy-ethonol ester of 2,4-D respectively.

While the concept of the ACR is simple to understand and implement, the use of ACRs has been widely criticized (Sloof *et al*., 1983; Baird *et al*., 1990; Lange *et al*., 1998; Warne, 1998; Calow, 2003). As with all acute to chronic extrapolation methods, the ACR method assumes that the toxic mechanisms in play are the same for both acute and chronic exposures (Warne, 1998). The validation of two extrapolation techniques, namely Linear Regression Analysis (LRA) (Mayer *et al*., 1994) and Multi-Factor Probit Analysis (MPA) (Lee *et al*., 1995) using fish toxicity data supports this assumption. The work of Baird *et al*. (1990) however does not. In a study of variation of response of different clones of *Daphnia magna* to cadmium chloride and a pesticide, Baird *et al*. (1990) showed interclonal variability in acute response but interclonal convergence in chronic response, concluding that acute stress in this species is dependant on genotypes and their ability to tolerate exposure to toxicants, while chronic response is related to fitness and less dependant on genotypes.

The definition of a chemical specific ACR (Rand, 1995) has broad interpretation, requiring only an acute measure and chronic measure of toxicity for the same species subjected to the same toxicant. If an LC_{50} and an NOEC toxicity measure are used as the acute and chronic measure respectively to derive the ACR, then the ACR should ideally be applied to acute LC_{50} toxicity measures to predict the NOEC toxicity measure (PNOEC). The ACR definition (Rand, 1995) however does not exclude the possibility of applying an ACR derived for example from an LC_{10} acute toxicity measure and an LOEC chronic toxicity measure to acute LC_{50} measures to predict chronic NOEC measures. In this study, the LC_{50} and NOEC measures are used as the acute and chronic measures respectively to derive an ACR, and ACR values are applied to acute LC_{50} measures to derive PNOEC measures.

The numerical value of an ACR is dependant on the biological responses measured in the respective acute and chronic tests (Warne, 1998). One may obtain different NOEC values in the same toxicity test, depending on the responses measured (Warne, 1998). Obviously the value of an ACR is dependant on the chronic NOEC value. In an analysis of ACRs on the ECETOC toxicity database, Lange *et al*. (1998) found that

one particular ACR had a value less than one (i.e. the chronic toxicity value was less conservative than the acute). Lange *et al*. (1998) found that drastically different biological responses were being measured in the acute and chronic toxicity tests used to derive the ACR and suggested that this is why the acute measure was more conservative than the chronic measure. The final numerical value of the NOEC is dependant on the concentrations chosen in the chronic toxicity test (Warne, 1998), and the statistical test chosen (Isnard *et al*., 2001) and this will affect the value of any ACR calculated using the NOEC.

The ACR method also does not take the effect of time on lethality into account. While it is generally accepted that acute toxicity tests last no longer than 96 hours, the exposure time for a chronic toxicity test is more dependant on the test organism and the biological response measured (Rand, 1995). A chronic toxicity test may run for the duration or part of a test organism's lifecycle or over the entire lifecycle of the test organism, and both toxicity tests can be termed chronic. Yet it is possible that the two tests will generate different tolerance information about the test organism. Younger life stages of aquatic vertebrates and invertebrates are generally more sensitive to toxicants than older life stages (Hutchinson *et al*., 1998). Therefore a chronic toxicity test that does not incorporate the most sensitive stages of the test organism's lifecycle will generate less conservative tolerance information, and an ACR generated with this chronic toxicity data would be too small.

The accuracy of the generic ACR is doubtful since tolerance of a particular species varies greatly with the toxicant it is exposed to (Warne, 1998), and different toxicants have different modes of action (Persoone *et al*., 1990). This fact is supported by the work of Sloof *et al*. (1983). In a comparison of the tolerance of 22 freshwater faunal and floral species to 15 chemical compounds, Sloof *et al*. (1983) found that interspecies tolerance to a particular chemical varied by as much as a factor of 9000. The authors concluded that one could not predict the response of a particular species to a toxicant by the response of another species to the same toxicant. The numerical values of chemical specific ACRs are highly variable indicating that fixed generic ACR values are inaccurate (Calow, 2003). Although ACRs are easy to understand and easy to use, there is no sound theoretical basis for their use (Warne, 1998).

2.1.2 Statistical methods for extrapolating (acute to chronic data)

2.1.2.1 Two – Step Linear Regression Analysis (LRA)

As the exposure time of a test organism to a toxicant increases, the toxicant concentration that causes an effect decreases in an asymptotic relationship (Warne, 1998). This however depends on the concentration of toxicant: a concentration that is too high will cause 100% mortality while a concentration that is too low will have no affect on the test organism. If LC_{50} values are plotted over time, the curve would show a decreasing LC_{50} concentration but the curve would level out somewhere above zero (Jooste and Rossouw, 2002) (Figure 2.1). A stabilised LC_{50} value would be called the threshold LC_{50} (Jooste and Rossouw, 2002) or incipient LC_{50} (Rand, 1995). The incipient/threshold LC_x measure is a concentration of a toxicant that is lethal to x % of the test organisms after a sufficiently long exposure time that acute lethal action has ceased (Rand, 1995). Jooste and Rossouw (2002) found that in toxicity tests, there is generally little change in LC_{50} measures after 10 days. Heming *et al*. (1989) found that the toxicity of the organo-chlorine pesticide methaxychlor to fish depended on the period of exposure with toxicity increasing with increasing exposure times. Importantly however, LC_{50} values tended to stabilise over time in all cases.

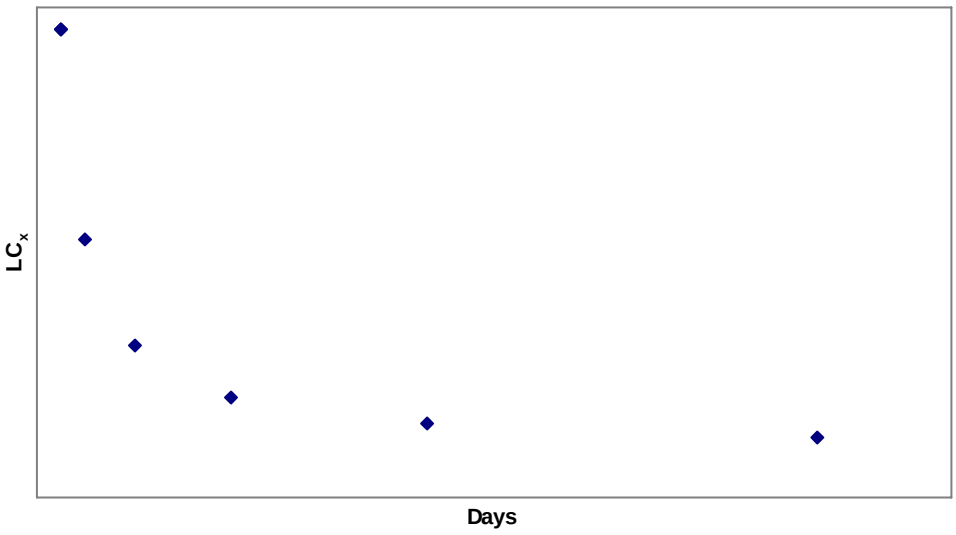


Figure 2.1 The above graphical representation is not based on any study but serves to demonstrate how the theoretical LC_x value in toxicity tests decreases in proportion to the duration of the toxicity test, up to a point were the LC_x value stabilises (threshold/incipient LC_{50}). Linear Regression Analysis (LRA) (Mayer *et al*., 1994) is based on this principle.

Two – Step Linear Regression Analysis (LRA) is an acute to chronic extrapolation method that considers concentration of toxicant, response to toxicant, and time-course of effect and involves two regression processes run sequentially (Mayer *et al*., 1994). The first regression process is the determination of LC_x values at each time period of observation within relevant toxicity tests. For example, using the data of one acute toxicity test, an LC_1 value can be generated for response data taken at 12 hours, another at 24 hours etc. The regression model chosen to obtain the LC_x value is the model that best fits the data. Mayer *et al*. (1994) however only used the Probit model to fit mortality data at this point i.e. the probit of accumulated percent mortality was plotted on the Y axis against the log concentration of the toxicant on the x axis. The first regression provides percent effect measures ($LC_{0,01}$ to LC_{99}) for particular observation times. The method assumes that any particular LC_x value will decrease with increasing observation time until a threshold is reached. The second regression aims to determine the duration at which a threshold will be reached and thereby provide an estimate of a time independent LC_x value or a threshold/incipient LC_x value. The second regression involves plotting the LC_x values as the dependant variable versus the reciprocal of observation time as the independent variable (Figure 2.2). The Y intercept of this regression gives an indication of the LC_x value over an infinite time period or chronic exposure period and is termed the threshold/incipient LC_x value.

The method makes two assumptions (Mayer *et al*., 1994):

1. Concentration response is a continuum in time.
2. The mode of action for lethality is similar under acute and chronic exposures.

Mayer *et al*. (1994) associated the threshold/incipient $LC_{0,01}$ with the Predicted No Observed Effect Concentration (PNOEC) and found that PNOECs generated with this method were accurate when compared to lethality Maximum Acceptable Toxicant Concentrations (MATCs) for fish species exposed to a variety of chemicals. The MATC is a chronic measure calculated as the geometric mean of the NOEC and LOEC (Rand, 1995). The LOEC or Lowest Observed Effect Concentration is the

lowest concentration in a concentration range that causes a detrimental effect to the test organisms that is significantly different to the control (Rand, 1995).

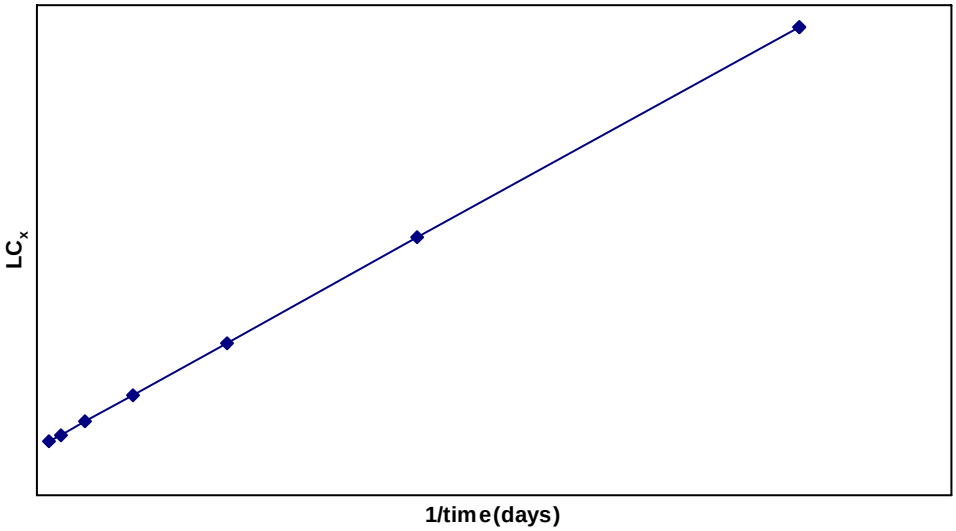


Figure 2.2 The second regression in Two-Step Linear Regression Analysis (LRA) (Mayer *et al*., 1994) involves plotting LC_x values over the reciprocal of time. The Y intercept indicates a threshold LC_x value (Mayer *et al*., 1994).

2.1.2.2 Multifactor Probit Analysis (MPA)

Multifactor Probit Analysis (MPA) is an acute to chronic toxicity data extrapolation method that simultaneously evaluates concentration, time and effect, and was first proposed by Lee *et al*. (1995). Unlike LRA, which utilises two regression models run sequentially, MPA uses two basic mathematical models of which there are three variations, each depending on the data transformations used (Lee *et al*., 1995). As stated by Lee *et al*. (1995), the first model assumes that the derivative of probit P (probability of death) with respect to C_x (Concentration of a toxicant causing a certain effect) is constant at each acute exposure time. This is termed 'Parellelism'.

Model 1: probit (P) = $\alpha + \beta C_x + \delta T_x$

The second model given by Lee *et al*. (1995) is the model for non-parallelism and allows for a change in slope as exposure time varies.

Model 2: probit (P) = $\alpha + \beta C_x + \delta T_x + \gamma CT_x$

Where P is probability of death, C_x may be concentration or log concentration, T_x is exposure time or the reciprocal of exposure time or the reciprocal of log exposure time. Other parameters, namely α, β, δ and γ are estimated via maximum likelihood techniques.

Lee *et al*. (1995) give six variant models based on the two main models.

Pair A based on log dose and log time:

A1: Probit P = $\alpha + \beta \log (C) + \delta \log(T)$

$C = 10^{(\text{Probit P} - \alpha - \delta \log(T))/ \beta}$

A2: Probit P = $\alpha + \beta \log (C) + \delta \log(T) + \gamma (\log(C) \log(T))$

$C = 10^{(\text{Probit P} - \alpha - \delta \log(T))/(\beta + \gamma \log(T))}$

Pair B based on log dose and reciprocal of time:

B1: Probit P = $\alpha + \beta \log (C) + \delta/T$

$C = 10^{(\text{Probit P} - \alpha - \delta/T)/ \beta}$

B2 : Probit P = $\alpha + \beta \log (C) + \delta/T + \gamma(\log(C)) / T$

$C = 10^{(\text{Probit P} - \alpha - \delta/T)/(\beta + \gamma / T)}$

Pair C based on log dose and reciprocal of log time:

C1: Probit P = $\alpha + \beta \log (C) + \delta/\log(T)$

$$C = 10^{\frac{(\text{Probit } P - \alpha - \delta/\log(T))}{\beta}}$$

C2: $\text{Probit } P = \alpha + \beta \log(C) + \delta/\log(T) + \gamma(\log(C)/\log(T))$

$$C = 10^{(\text{Probit } P - \alpha - \delta/\log(T)) / (\beta + \gamma / \log(T))}$$

Associated with each model is a 'goodness of fit' statistic that reflects the degree of discrepancy between the actual responses in the data and the responses estimated from the model. The model with the best 'goodness of fit' statistic is chosen to obtain acute to chronic extrapolation results. Making 'C' (concentration of toxicant) the subject of the formula will give the concentration of a toxicant that will cause a particular effect (0.01 to 99.9 percent effect). The degree of effect is specified by Probit P and T (time of exposure) and must be entered at the modeller's discretion. Some idea of the lifecycle of the test organism or the time taken for mortality to stabilise (threshold time) in a toxicity test (Jooste and Rossouw, 2002) is needed to enter a meaningful value for T in the models.

Concentrations causing a small degree of effect can be estimated such as 0,01% - 10 % effect. Lee *et al*. (1995) chose the 0,01 % effect value to be representative of a Predicted No Observed Effect Concentration (PNOEC) that is the predicted value of the No Observed Effect Concentration (NOEC). The 0,01 % percent effect was chosen as it is a small effect value close to zero. Lee *at al*. (1995) applied the method to fish acute toxicity data for a variety of chemicals and found that generated PNOECs were within a factor of two of chronic MATC toxicity measures for the same chemicals 70% of the time.

2.1.3 Aims of chapter 2

The aim of this chapter is to explore acute to chronic toxicity data extrapolation techniques. Three methods of converting acute toxicological data to chronic toxicological data will be assessed in terms of conservativeness, i.e. the methods will be ranked in terms of how protective they are. Two statistical methods i.e. Two Step Linear Regression and Multiple Factor Probit Analysis as well as an assessment factor method i.e. Acute to Chronic Ratios (ACRs) will be applied to a set of freshwater

aquatic invertebrate acute toxicological data where the toxicants are NaCl and Na_2SO_4 and the invertebrate species are indigenous to South Africa.

2.2 Materials and Methods

2.2.1 Acute toxicity data

As Acute to Chronic Ratios, Two-Step Linear Regression and Multifactor Probit Analysis extrapolate chronic toxicity data from acute toxicity data, the initial requirement for the process is suitable acute toxicity data. In this case, acute toxicity data were obtained from the Institute for Water Research (IWR) - Unilever Centre for Environmental Water Quality (UCEWQ) toxicity database. None of these tests were conducted as part of this study. The database contains acute and short-term chronic toxicity data for a range of indigenous aquatic invertebrates exposed to a range of toxicants (Scherman *et al*., 2002; Palmer *et al*., in press). The short-term chronic toxicity tests on the UCEWQ toxicity database measure survival and have an exposure duration of 10 days. Treatments in the short-term chronic tests were chosen for regression analysis. All toxicity data on the UCEWQ toxicity database data are of a high standard and water quality variables are routinely measured (pH, Dissolved Oxygen, Electrical Conductivity, Total Dissolved Salts, nitrates and nitrites, phosphates and ammonia). Additionally, in the majority of toxicity tests run by the IWR/UCEWQ, water samples were collected from all treatments for the analysis of concentrations of various trace metals. Toxicity tests were run in laboratories with controlled temperatures (±1°C) with minimum and maximum air temperatures, as well as experimental temperatures, of all treatments measured daily. Toxicant salts used were pure grade salts prepared for laboratory use. Acute and short-term chronic data where NaCl and Na_2SO_4 were used as toxicants were collated for use in the extrapolations (Tables 2.1 and 2.2). The data show that responses among and within species to NaCl and Na_2SO_4 are variable (Figures 2.3 – 2.6).

Table 2.1 A summary of acute and short - term chronic toxicity data for NaCl contained on the IWR/UCEWQ toxicity database and used for acute to chronic toxicity data extrapolation. The 'River' column indicates where test organisms were collected for the respective toxicity test. The 'Experimental System' column indicates the exposure method used in the respective toxicity test (see Rand, 1995). D.T.W: Dechlorinated Tap Water.

Species	**Common Name**	**River**	**Duration (hours)**	**Replicate**	**Diluent**	**Experimental System**	**Measured Endpoint**	**LC_{50} (mg/L)**
Adenophlebia auriculata	Mayfly	Palmiet River	240	1	D.T.W	Re-circulating	Lethality	5630
Adenophlebia auriculata	Mayfly	Palmiet River	240	2	D.T.W	Re-circulating	Lethality	5394
Adenophlebia sylvatica	Mayfly	Kat River	120	1	D.T.W	Re-circulating	Lethality	5979
Adenophlebia sylvatica	Mayfly	Kat River	192	2	D.T.W	Re-circulating	Lethality	4502
Afronurus barnardi	Mayfly	Molenaars River	168	1	River Water	Re-circulating	Lethality	3503
Afronurus barnardi	Mayfly	Molenaars River	168	1	River Water	Re-circulating	Lethality	3063
Afronurus barnardi	Mayfly	Kat River	240	1	D.T.W	Re-circulating	Lethality	3186
Afronurus barnardi	Mayfly	Kat River	240	1	D.T.W	Re-circulating	Lethality	3157
Afronurus peringueyi	Mayfly	Vaal River	96	1	N/A	Re-circulating	Lethality	6290
Afronurus peringueyi	Mayfly	Bushman's River	240	1	D.T.W	Re-circulating	Lethality	1770
Afronurus peringueyi	Mayfly	Keurbooms River	240	1	Rain Water	Re-circulating	Lethality	4002
Afronurus peringueyi	Mayfly	Keurbooms River	240	2	Rain Water	Re-circulating	Lethality	3523
Baetid sp.	Mayfly	Palmiet River	240	1	D.T.W	Re-circulating	Lethality	3542
Baetid sp.	Mayfly	Palmiet River	240	2	D.T.W	Re-circulating	Lethality	3642
Baetis harrisoni	Mayfly	Balfour River	96	1	D.T.W	Re-circulating	Lethality	1689
Burnupia stenochorias	Limpit	Kat River	96	1	D.T.W	Re-circulating	Lethality	3653
Trichoptera sp.	Caddisfly	Drager Dam	96	1	D.T.W	Static	Lethality	7668
Trichoptera sp.	Caddisfly	Howisons Poort	96	1	D.T.W	Static	Lethality	5803
Trichoptera sp.	Caddisfly	Drager Dam	96	1	D.T.W	Static	Lethality	5621
Caridina nilotica	Shrimp	Laboratory Culture	48	1	D.T.W	Static	Lethality	5955

Table 2.1 continued A summary of acute and short - term chronic toxicity data for NaCl contained on the IWR/UCEWQ toxicity database and used for acute to chronic toxicity data extrapolation. The 'River' column indicates where test organisms were collected for the respective toxicity test. The 'Experimental System' column indicates the exposure method used in the respective toxicity test (see Rand, 1995). D.T.W: Dechlorinated Tap Water.

Species	**Common Name**	**River**	**Duration (hours)**	**Replicate**	**Diluent**	**Experimental System**	**Measured Endpoint**	**LC_{50} (mg/L)**
Caridina nilotica	Shrimp	Laboratory Culture	48	1	D.T.W	Static	Lethality	4450
Caridina nilotica	Shrimp	Laboratory Culture	48	1	D.T.W	Static	Lethality	5979
Caridina nilotica	Shrimp	Laboratory Culture	48	1	D.T.W	Static	Lethality	5487
Caridina nilotica	Shrimp	Laboratory Culture	96	1	D.T.W	Static	Lethality	8568
Cloeon virgiliae	Mayfly	Drager Dam	96	1	D.T.W	Static	Lethality	5218
Enallagma sp.	Damselfly	Drager Dam	84	1	D.T.W	Static	Lethality	21608
Enallagma sp.	Damselfly	Drager Dam	96	1	D.T.W	Static	Lethality	18299
Euthraulus elegans	Mayfly	Breede River	60	3	D.T.W	Re-circulating	Lethality	8249
Euthraulus elegans	Mayfly	Vaal River	96	1	D.T.W	Re-circulating	Lethality	6899
Euthraulus elegans	Mayfly	Vaal River	96	1	D.T.W	Re-circulating	Lethality	7625
Euthraulus elegans	Mayfly	Kat River	96	1	D.T.W	Static	Lethality	7270
Euthraulus elegans	Mayfly	Kat River	96	1	D.T.W	Re-circulating	Lethality	8957
Euthraulus elegans	Mayfly	Breede River	168	2	D.T.W	Re-circulating	Lethality	5831
Euthraulus elegans	Mayfly	Keurbooms River	240	1	Rain Water	Re-circulating	Lethality	2212
Euthraulus elegans	Mayfly	Bushman's River	240	1	D.T.W	Re-circulating	Lethality	6466
Euthraulus elegans	Mayfly	Kat River	240	1	D.T.W	Re-circulating	Lethality	4342
Euthraulus elegans	Mayfly	Kat River	240	2	D.T.W	Re-circulating	Lethality	3429
Euthraulus elegans	Mayfly	Kat River	240	3	D.T.W	Re-circulating	Lethality	4890
Euthraulus elegans	Mayfly	Kat River	240	1	D.T.W	Static Renewal	Lethality	4744

Table 2.1 continued A summary of acute and short - term chronic toxicity data for NaCl contained on the IWR/UCEWQ toxicity database and used for acute to chronic toxicity data extrapolation. The 'River' column indicates where test organisms were collected for the respective toxicity test. The 'Experimental System' column indicates the exposure method used in the respective toxicity test (see Rand, 1995). D.T.W: Dechlorinated Tap Water.

Species	**Common Name**	**River**	**Duration (hours)**	**Replicate**	**Test Medium**	**Experimental System**	**Measured Endpoint**	**LC_{50} (mg/L)**
Euthraulus elegans	Mayfly	Bushman's River	240	1	D.T.W	Re-circulating	Lethality	5230
Euthraulus elegans	Mayfly	Kat River	240	1	D.T.W	Static Renewal	Lethality	2786
Plea pullula	Backswimmer	Drager Dam	96	1	D.T.W	Static	Lethality	7616
Tricorythus discolor	Mayfly	Breede River	96	3	D.T.W	Re-circulating	Lethality	3906
Tricorythus discolor	Mayfly	Breede River	168	2	River Water	Re-circulating	Lethality	3951
Tricorythus discolor	Mayfly	Breede River	168	3	River Water	Re-circulating	Lethality	2098
Tricorythus discolor	Mayfly	Breede River	168	1	D.T.W	Re-circulating	Lethality	1888
Tricorythus discolor	Mayfly	Breede River	168	2	D.T.W	Re-circulating	Lethality	906
Tricorythus discolor	Mayfly	Kat River	180	1	D.T.W	Re-circulating	Lethality	3167
Tricorythus discolor	Mayfly	Kat River	192	1	D.T.W	Static Renewal	Lethality	3012
Tricorythus discolor	Mayfly	Breede River	216	1	River Water	Re-circulating	Lethality	1130
Tricorythus discolor	Mayfly	Kat River	240	1	D.T.W	Re-circulating	Lethality	2701
Tricorythus discolor	Mayfly	Kat River	240	2	D.T.W	Re-circulating	Lethality	3354
Tricorythus discolor	Mayfly	Mooi River	240	1	D.T.W	Re-circulating	Lethality	1277
Tricorythus discolor	Mayfly	Mooi River	240	2	D.T.W	Re-circulating	Lethality	1873
Tricorythus discolor	Mayfly	Mooi River	240	3	D.T.W	Re-circulating	Lethality	2358
Tricorythus tinctus	Mayfly	Sabie River	96	1	Rain Water	Re-circulating	Lethality	1689
Tricorythus tinctus	Mayfly	Sabie River	240	1	Rain Water	Re-circulating	Lethality	839

Table 2.2 A summary of acute and short - term chronic toxicity data for Na_2SO_4 contained on the IWR/UCEWQ toxicity database and used for acute to chronic toxicity data extrapolation. The 'River' column indicates where test organisms were collected for the respective toxicity test. The 'Experimental System' column indicates the exposure method used in the respective toxicity test (see Rand, 1995). D.T.W: Dechlorinated Tap Water.

Species	**Common name**	**River**	**Duration (hours)**	**Replicate**	**Test Medium**	**Experimental System**	**Measured Endpoint**	**LC_{50} (mg/L)**
Tricorythus tinctus	Mayfly	Sabie River	96	1	River Water	Re-circulating	Lethality	2757
Tricorythus tinctus	Mayfly	Sabie River	288	1	River Water	Re-circulating	Lethality	432
Tricorythus tinctus	Mayfly	Sabie River	240	1	River Water	Re-circulating	Lethality	430
Adenophlebia auriculata	Mayfly	Palmiet River	96	1	D.T.W	Re-circulating	Lethality	10379
Adenophlebia auriculata	Mayfly	Palmiet River	96	1	D.T.W	Re-circulating	Lethality	6303
Adenophlebia auriculata	Mayfly	Palmiet River	96	1	D.T.W	Re-circulating	Lethality	8073
Afroptilum sudafricanum	Mayfly	Palmiet River	96	1	D.T.W	Re-circulating	Lethality	4651
Afroptilum sudafricanum	Mayfly	Palmiet River	96	1	D.T.W	Re-circulating	Lethality	2755
Afroptilum sudafricanum	Mayfly	Palmiet River	60	1	D.T.W	Re-circulating	Lethality	2382
Afroptilum sudafricanum	Mayfly	Palmiet River	96	1	D.T.W	Re-circulating	Lethality	3096
Oligoneuriopsis lawrencei	Mayfly	Balfour River	96	1	D.T.W	Re-circulating	Lethality	752
Plea pullula	Backswimmer	Drager Dam	96	1	D.T.W	Static	Lethality	9355
Belostomatidae sp.	Water Bug	Howison's Poort	96	1	D.T.W	Static	Lethality	7630
Cloeon virgiliae	Mayfly	Drager Dam	96	1	D.T.W	Static	Lethality	4089
Cloeon virgiliae	Mayfly	Howison's Poort	96	1	D.T.W	Static	Lethality	6028
Euthraulus elegans	Mayfly	Kat River	96	1	D.T.W	Re-circulating	Lethality	10165
Euthraulus elegans	Mayfly	Balfour River	96	1	D.T.W	Re-circulating	Lethality	8580
Burnupia stenochorias	Limpit	Kat River	96	1	D.T.W	Re-circulating	Lethality	4580
Burnupia stenochorias	Limpit	Kat River	96	1	D.T.W	Re-circulating	Lethality	5282

Table 2.2 A summary of acute and short - term chronic toxicity data for Na_2SO_4 contained on the IWR/UCEWQ toxicity database and used for acute to chronic toxicity data extrapolation. The 'River' column indicates where test organisms were collected for the respective toxicity test. The 'Experimental System' column indicates the exposure method used in the respective toxicity test (see Rand, 1995). D.T.W: Dechlorinated Tap Water.

Species	**Common Name**	**River**	**Duration (hours)**	**Replicate**	**Test Medium**	**Experimental System**	**Measured Endpoint**	**LC_{50} (mg/L)**
Afronurus barnardi	Mayfly	Kat River	96	1	D.T.W	Re-circulating	Lethality	5924
Planaria sp.	Flatworm	Kat River	240	1	D.T.W	Static Renewal	Lethality	9177
Enallagma sp	Damselfly	Drager Dam	96	1	D.T.W	Static	Lethality	24149
Enallagma sp	Damselfly	Drager Dam	96	1	D.T.W	Static	Lethality	26224
Trichoptera sp.	Caddisfly	Drager Dam	96	1	D.T.W	Static	Lethality	11345
Trichoptera sp.	Caddisfly	Drager Dam	96	1	D.T.W	Static	Lethality	9803
Trichoptera sp.	Caddisfly	Howison's Poort	96	1	D.T.W	Static	Lethality	8766
Baetis harrisoni	Mayfly	Balfour River	96	1	D.T.W	Re-circulating	Lethality	3805
Caridina nilotica	Shrimp	Mpisini Stream	240	1	D.T.W	Re-circulating	Lethality	4955
Caridina nilotica	Shrimp	Laboratory Culture	240	1	D.T.W	Re-circulating	Lethality	3249
Caridina nilotica	Shrimp	Laboratory Culture	96	1	D.T.W	Static	Lethality	6820
Caridina nilotica	Shrimp	Laboratory Culture	240	1	D.T.W	Re-circulating	Lethality	3149
Caridina nilotica	Shrimp	Laboratory Culture	48	1	D.T.W	Static	Lethality	5989
Caridina nilotica	Shrimp	Laboratory Culture	48	1	D.T.W	Static	Lethality	7002
Caridina nilotica	Shrimp	Laboratory Culture	48	1	D.T.W	Static	Lethality	5734
Caridina nilotica	Shrimp	Laboratory Culture	48	1	D.T.W	Static	Lethality	5477
Tricorythus discolor	Mayfly	Olifants River	252	1	D.T.W	Re-circulating	Lethality	1550
Tricorythus discolor	Mayfly	Olifants River	144	2	D.T.W	Re-circulating	Lethality	2589
Tricorythus discolor	Mayfly	Olifants River	120	3	D.T.W	Re-circulating	Lethality	4165

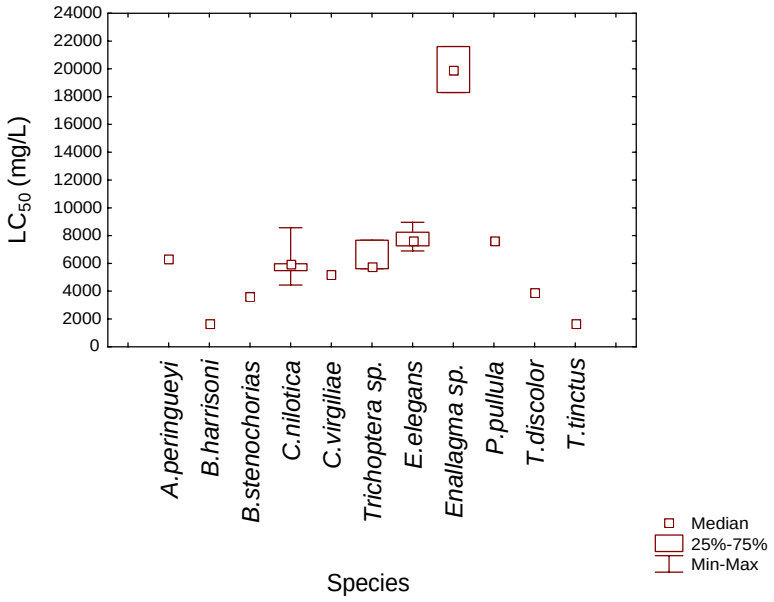


Figure 2.3 Variability of inter-species and intra - species tolerance to NaCl acute (96 hour) exposure according to toxicity data in the IWR/UCEWQ database.

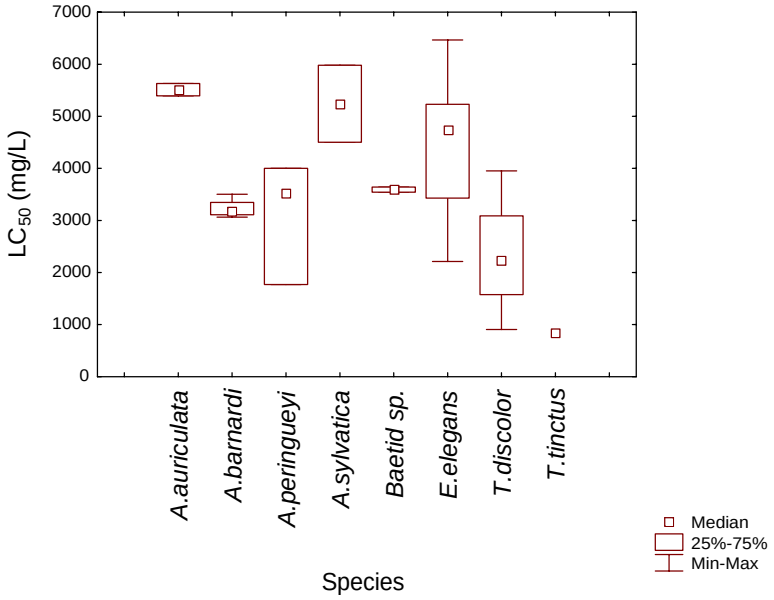


Figure 2.4 Variability of inter - species and intra - species tolerance to NaCl short-term chronic (> 96 hour) exposure according to toxicity data in the IWR/UCEWQ database.

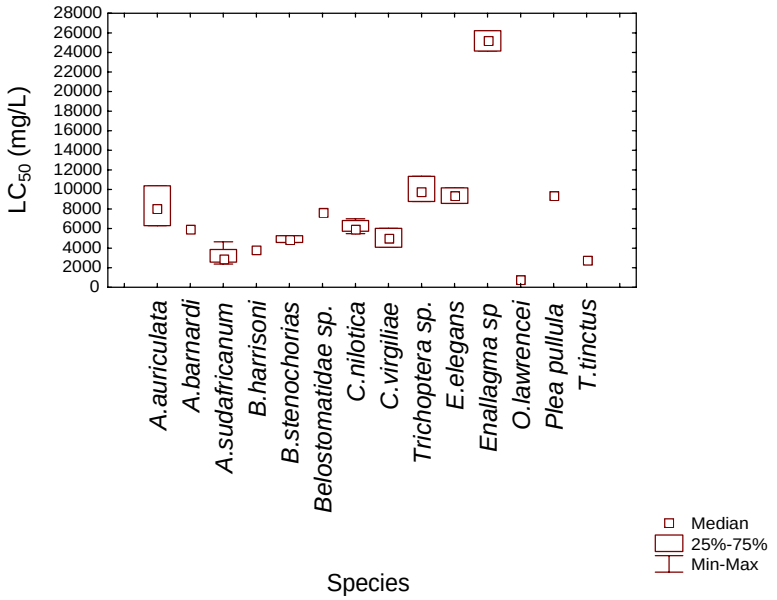


Figure 2.5 Variability of inter - species and intra - species tolerance to Na_2SO_4 acute (96 hour) exposure according to toxicity data in the IWR/UCEWQ database.

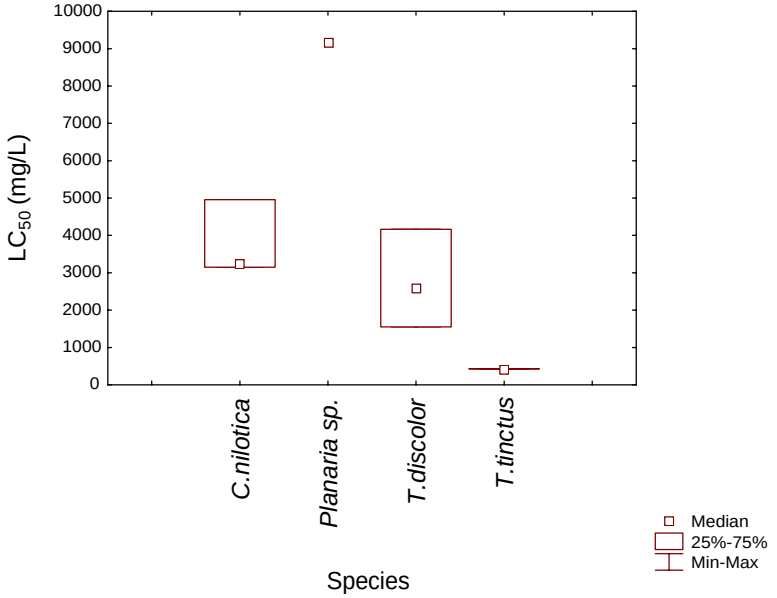


Figure 2.6 Variability of inter - species and intra - species tolerance to Na_2SO_4 short – term chronic exposure (> 96 hour) according to toxicity data in the IWR/UCEWQ database.

Toxicity tests for the salts NaCl and Na_2SO_4 represented on the database used test organisms from various rivers of South Africa. Rivers in the Eastern Cape included the Kat, Balfour and Palmiet Rivers. Rivers from the Kwazulu - Natal region included the Mooi and Bushman's River. Toxicity data were obtained from the Breede and Keurbooms Rivers in the Western Cape. Rivers in the Gauteng region included the Vaal River and from the Northern Province region the Sabie and Olifants Rivers. The toxicological data therefore represents the salinity tolerance of indigenous macro-invertebrates on a national level.

2.2.2 Preparation of toxicity test data

Toxicological data were grouped by toxicant and organism for analysis. Measured electrical conductivity (mS/m) (from known nominal salt exposure concentrations (mg/L)) and response data for each concentration, at all measured time periods during the acute tests, were recorded into a spreadsheet. Average measured electrical conductivity for each nominal salt exposure concentration was calculated for each observation period and used for analysis. Records within the spreadsheet were grouped by experimental identification number, replicate and observation period for analysis. Most tests consisted of only one replicate since regression analysis is the preferred IWR/UCEWQ form of statistical analysis for acute toxicity test data (Tables 2.1 and 2.2). Acute tests (≤ 96 hours exposure) were rejected where there was greater than 10% mortality in the control and short - term chronic tests (96 hours - 10 days exposure) were rejected where there was greater than 20% mortality in the control (Rand, 1995). Even when toxicity tests had unacceptably high control mortalities at the time the test ended, survival observations would still have been used at observation times during the test when control mortalities were still acceptable (Table 2.1 and 2.2).

2.2.3 Data Analysis

2.2.3.1 Acute to chronic ratios (ACRs)

The calculation of an ACR is defined as an acute toxicity value divided by a chronic toxicity value (Warne, 2001). Acute toxicity endpoints are usually represented by an LC_{50} (or EC_{50}) value and chronic endpoints are usually represented by an NOEC. Of course, the same unit of measurement for the acute and chronic endpoints must be

used. While acute toxicity tests for multi-celled animals are defined as tests where the exposure is between 24 and 96 hours duration, chronic tests for multi-celled organisms are defined as tests where the time of exposure is greater than the maximum time for acute exposure (Rand, 1995; Warne, 2001). In this regard, short-term chronic tests on the UCEWQ toxicity database of 10 days duration were considered to fall into the category of chronic tests, and therefore data in these tests were analyzed to obtain a chronic NOEC value to calculate an ACR. A NOEC value for a particular short-term chronic toxicity test was obtained by running a parametric ANOVA on mortality data where there were at least three replicated concentration ranges, followed by a parametric post-hoc test to determine which concentrations were statistically different from the control.

Parametric statistical methods assume that datasets have equal variance and that the data follows a normal distribution (Crane *et al*., 2000). An ANOVA however is known to be fairly robust in this respect (Crane *et al*., 2000). If, after various data transformations were performed on the data, the requirements for parametric statistics were still not met, non-parametric statistics were used. Only short –term chronic data where mortality in the control was 20 % or less for any of the replicates were considered (Cooney, 1995).

In the compilation of ACRs for the derivation of the ANZECC and ARMCANZ (2000) trigger values, acute and chronic endpoints for the same species exposed to the same toxicant and values presented in the same publication or at least conducted in the same laboratory were used (Warne, 2001). In this regard, acute and chronic toxicity endpoints on the UCEWQ database were considered for the calculation of an ACR if they were obtained in the same laboratory and the same methodology was used.

In addition, an ACR for *C.nilotica* exposed to NaCl and Na_2SO_4 was calculated from the results of the chronic testing on this species outlined in Chapter 3. Acute to Chronic Ratios (ACRs) were compiled for NaCl and Na_2SO_4 using the results of the chronic tests and acute tests contained on the IWR/UCEWQ toxicity database. Initially, the suitability of the acute data to fit the Probit model was determined using

Probit Program Version 1.5. (USEPA, 1993). If the model did not fit the data adequately, as indicated by a chi^2 value, or if the slope of the regression line was not significant, the data were fitted to linear regression models in Statistica Version 6 (StatSoft, 2002), either with no data transformation, or using the log data transformation, where $x' = \log_{10} x + 1$, x' represents the transformed data and x represents the untransformed data. The LC_{50} value from the model that gave the highest R^2 was chosen. The numerical value of the ACR for each salt was determined by dividing the geometric mean of the 96-hour LC_{50} values obtained from acute toxicity tests by the NOEC determined in the chronic toxicity test. The derivation of ACRs was done at the species level i.e. acute and chronic toxicity tests used to derive an ACR were for the same species and using the same toxicant. The geometric mean was chosen to lessen the effect of skewed data (Warne *pers.comm.*, 1994).

As is consistent with use by Warne (2001), if only one ACR for a particular chemical was found, that ACR was used for all acute toxicity data where that chemical is the toxicant, regardless of the species or type of test animal. If more than one ACR value was obtained, the geometric mean of the ACR values was used (Warne, 2001).

All toxicity data available on the UCEWQ database are for aquatic macroinvertebrates. One could expect that ACRs obtained from these data and applied to invertebrate acute data are more accurate than applying ACR values obtained from vertebrate or algal toxicity data.

Only 96 hour toxicological data in an LC_{50} measured value were considered for conversion using the ACRs. Short - term chronic data were not extrapolated as these data were considered as chronic data in the calculation of ACRs. Although short – term chronic data were used with the LRA and MPA extrapolation implementations, these methods incorporate the duration of exposure into predicted chronic calculations, while ACRs do not. Therefore PNOEC values generated with the ACR method are comparable with values obtained with LRA and MPA extrapolations. If multiple LC_{50} values were available for any particular species, the geometric mean of the LC_{50} values were calculated. For a particular species, all acute toxicological tests were required to have the same experimental design to be considered. For example,

toxicological data where the experimental system design used was of a re-circulating design were not considered together with toxicological data where the experimental design used was static renewal, for any particular species. This was to prevent avoidable variance between LC_{50} values because of differing experimental systems.

Statistica Version 6 (StatSoft, 2002) was the statistical software used for all statistical analyses.

2.2.3.2 Two – Step Linear Regression Analysis (LRA)

Probit (USEPA, 1993) or least – square linear regression equations with data transformations other than those used in Probit were generated for each time period in each test. Probit data were rejected where the data did not fit the model (as indicated by a chi^2 value) or where the slope of the regression generated by Probit analysis was not significant. Least – square regression was performed using Statistica Version 6 (StatSoft, 2002) when the Probit model did not fit the data, with the following being regressed:

Regression equation 1:
% mortality = Y intercept + slope * toxicant concentration

or

Regression equation 2:
log(% mortality + 1) = Y intercept + slope * log(toxicant concentration + 1).

The LC_x value from the regression giving the highest R^2 value was selected from regression one and two. Mayer *et al*. (1994) originally extrapolated low effect values from $LC_{0,01}$ to LC_{10} as possible PNOECs. However, low LC_x values of less than 10% have been found to be model dependant (Moore and Caux, 1997; Isnard *et al*., 2001) although this may depend on the quality of toxicity data. Confidence intervals can also be excessively large for low effects of five percent or less (Clarke *et al*., 2002). It may be more statistically rigorous to extrapolate LC_{50} values, using LRA, where confidence intervals are smaller and subsequently apply an application or safety factor

to the threshold LC_{50} to obtain a PNOEC. In the derivation of the Australian and New Zealand Water Quality Guidelines, chronic time scale LC_{50}s for metal toxicants were converted to NOEC values by applying a safety factor of 5 (Warne, 2001). This safety factor was only applied to chronic time LC_{50}s for metal data, as there were sufficient NOEC values available for other toxicants (Warne, 2001). Isnard *et al*. (2001) in a comparison of NOEC and EC_x values produced in chronic toxicity testing using hypothesis and regression statistics respectively, found that the median EC_{50}/NOEC ratio was 2,3. A safety factor of 5 is therefore likely to be protective. Therefore, in this study, asymptotic LC_{50} values were extrapolated using LRA and divided by 5 to provide an estimate of a PNOEC.

$LC_{0.01}$, $LC_{0.1}$, LC_1, LC_5, $LC_{10\ and}$ LC_{50} values were generated from the valid Probit equations or from regression one or two. The least – square approach was limited to interpolation situations as recommended by Clarke *et al*. (2002). Low LC_x values ($LC_{0.01} - LC_{10}$) were selected, as they are low toxicity endpoints and are suggested to represent chronic toxicity endpoints (Mayer *et al*., 1994; Lee *et al*., 1995; Scherman *et al*., 2003). LC_{50} values were also selected because of high confidence and model independence at this value. The LC_x values grouped by toxicant and organism, were plotted over time to assess whether to use or reject data. Data that did not show increasing toxicity with time were rejected. Values for 1/time, 1/log(time) and log(LC_x) were generated for use as variables within the regressions.

In Statistica (StatSoft, 2002), R^2 values for the following regressions were established:

1. $LC_x = y + m/t$.
2. $\log_{10}LC_x = y + m/t$.
3. $\log_{10}LC_x = y + m/\log_{10}t$.

Where:

LC_x represents a toxicity value ranging from $LC_{0,01} - LC_{10\ and}$ LC_{50} generated from mortality observations taken at time t.

y represents the y-intercept of the regression.

m represents the slope of the regression line.

t represents the observation time.

The regression that gave the highest R^2 value was chosen and the regression line plotted in Statistica (StatSoft, 2002). The y – intercept of each regression line gave the magnitude of an indefinite LC_x value, for comparison with generated ACR and MPA values.

2.2.3.3 Multiple Factor Probit Analysis (MPA)

The models for Multifactor Probit Analysis were run in Microsoft Excel (Microsoft, 2002). Only acute and short-term chronic toxicological data where there were at least 5 concentrations and four observation times were used, as recommended by Lee *et al*. (1995). The format of the spreadsheet follows the format recommended by Caux and Moore (1996) for running models in Microsoft Excel (Microsoft, 2002). The spreadsheet has a data entry section (Figure 2.7), a summary section (Figure 2.8) and a section for each of the specified six models (Figure 2.9). A goodness of fit statistic is calculated as $\Sigma_{(i = 1- n)} (O_i – E_i)^2/E_i$ (Rand , 1995) where O_i is the observed effect, E_i is the expected effect calculated from the model and n is the number of data entries for the acute toxicological data available. The parameters α, β, δ and γ for the models are estimated via maximum likelihood techniques using SOLVER in Excel (Microsoft, 2002) (Figure 2.10). The threshold exposure time to be used in the model was estimated by plotting low LC_x data in the range of $LC_{0,01} – LC_{10}$ for a particular organism exposed to a particular toxicant against time. The time taken for the LC_x to stabilise was taken as the threshold time. This analysis is run in Statistica Version 6 (StatSoft, 2002). Jooste and Rossouw (2002) found that LC_{50} values generally stabilised after 10 days. Jooste and Rossouw (2002) extrapolated acute LC_{50} values to two weeks (336 hours) as a precautionary measure, but short-term chronic (10 day) toxicity tests in the IWR/UCEWQ database generally showed LC_{50} stabilisation before 10 days. Therefore, where there were not enough LC_x data available to estimate a threshold time, a threshold time of 240 hours was assumed. Although this method reports on the full range of LC_x values, low LC_x values in the range of $LC_{0,01} – LC_{10}$ are reported as possible PNOEC values (Lee *et al*., 1995). In addition, the LC_{50} values were reported because of the high confidence level (Clarke *et al*., 2002) and

model independence of this value (Moore and Caux, 1997; Isnard *et al.*, 2001). The reported LC_{50} values were divided by a safety factor of 5 to estimate a PNOEC value (Warne, 2001).

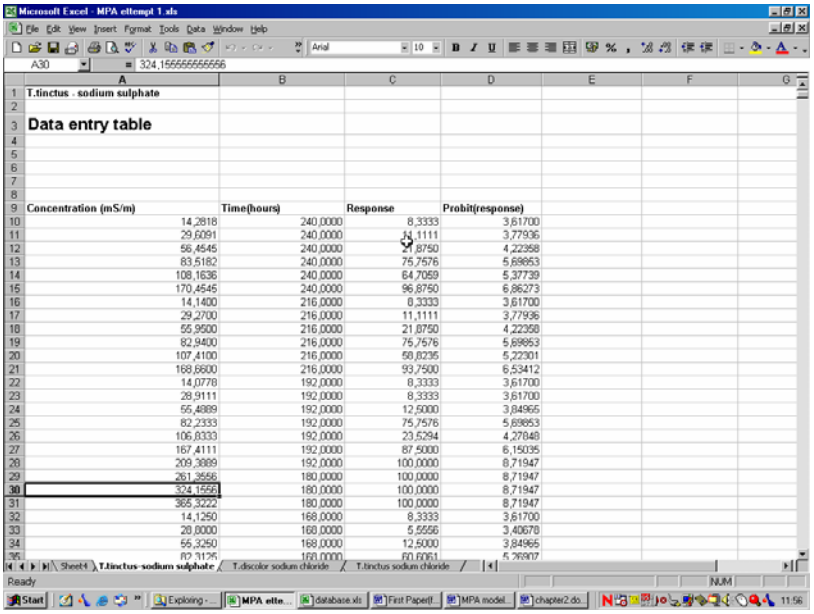


Figure 2.7 Data entry section for the Multifactor Probit Analysis (Lee *et al.*, 1995) implementation in Microsoft Excel (Microsoft, 2002).

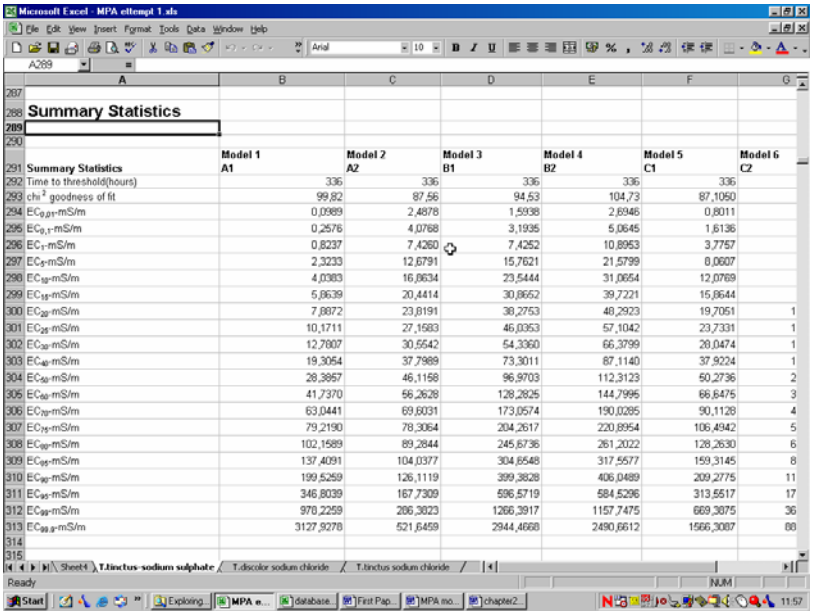


Figure 2.8 Summary section for the Multifactor Probit Analysis (Lee *et al.*, 1995) implementation in Microsoft Excel (Microsoft, 2002).

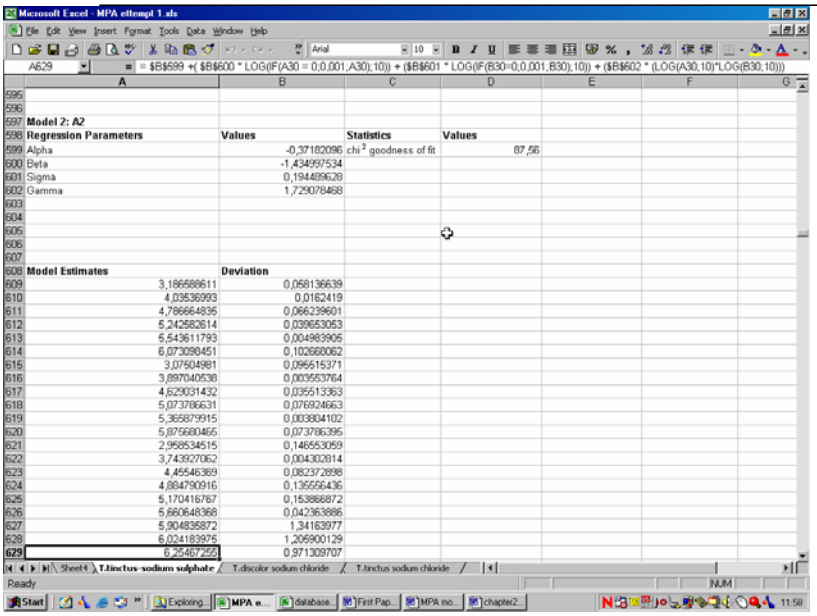


Figure 2.9 Model estimate section for the Multifactor Probit Analysis (Lee *et al.*, 1995) implementation in Microsoft Excel (Microsoft, 2002).

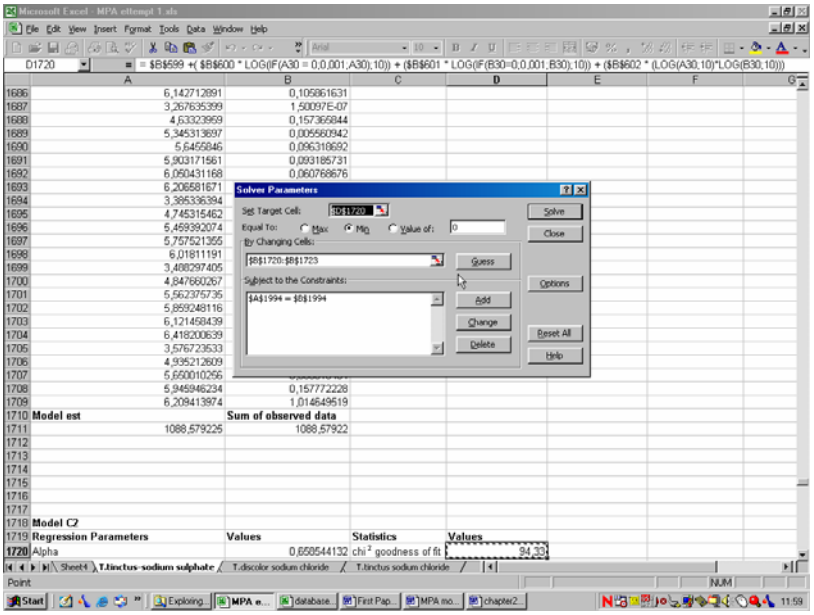


Figure 2.10 Using SOLVER in the Multifactor Probit Analysis (Lee *et al.*, 1995) implementation in Microsoft Excel (Microsoft, 2002).

2.3 Results

2.3.1 Extrapolations using Acute to Chronic Ratios

Four acute toxicity tests of 48 hours where NaCl was the toxicant using *Caridina nilotica* (Decapoda: Atyidae) juveniles (< 7 days of age) were available on the UCEWQ toxicity database (Table 2.1). Only one acute test of 96 hours was available on the database using *C.nilotica* adults. It was decided to use the 48 hour juvenile toxicity tests as there were more data available and juveniles were also used in the chronic toxicity tests. The results of the 96 hour acute test were not used with the 48 hours tests because of the discrepancy in the exposure duration. The four 48 hour tests yielded LC_{50} values of 5979 mg/L, 5955 mg/L, 4450 mg/L and 5487 mg/L with a geometric mean of 5430 mg/L. The geometric mean was divided by the NaCl experimental chronic NOEC of 1900 mg/L for *C.nilotica* (Chapter 3) for an ACR of 2.9. Although NOEC values were obtained for different biological responses in the NaCl chronic test, 1900 mg/L was the most common NOEC measured.

Four acute toxicity tests of 48 hours where Na_2SO_4 was the toxicant using *C.nilotica* juveniles (< 7 days of age) were available on the UCEWQ toxicity database (Table 2.2). The four 48 hour tests yielded LC_{50} values of 5989 mg/L, 7002 mg/L, 5734 mg/L and 5477 mg/L with a geometric mean of 6024 mg/L. The geometric mean was divided by the Na_2SO_4 experimental chronic NOEC of 1900 mg/L for *C.nilotica* (Chapter 3) for an ACR of 3.17. Although NOEC values were obtained for different biological responses in the Na_2SO_4 chronic test, 1900 mg/L was the most common NOEC measured.

Short-term chronic mortality data on the IWR/UCEWQ toxicity database were screened for suitability as chronic data to produce an NOEC value. Short-term chronic toxicity tests involving *Euthraulus elegans* (Ephemeroptera: Leptophlebiidae) exposed to NaCl, *Tricorythus discolor* (Ephemeroptera: Tricorythidae) exposed to NaCl and *T.discolor* exposed to Na_2SO_4 were found where each test had three replicated concentration ranges. Unfortunately, only the test involving *E.elegans* did not have unacceptable control mortality in any of the replicated concentration ranges. Control mortalities in each replicate were 0, 0 and 6.5 % for replicates one, two and

three respectively. The number of animals used in the treatments ranged from 26 to 38. The 4000 mg/L treatment was identified as a possible LOEC in the test (Figure 2.11). A Levene's test indicated that the data had unequal variance ($p < 0,05$), and a Shapiro-Wilk test for normality gave a W statistic of 0.83, indicating that the data were not normally distributed. The following data transformations were performed on the data (ZAR, 1974):

The logarithmic transformation where $X' = \log_{10}(X + 1)$.
The arcsine transformation where $X' = \arcsin X^{1/2}$.
The square root transformation where $X' = (X + 0.5)^{1/2}$.

X represents the original accumulated % mortality data and X' represents the transformed accumulated % mortality data.

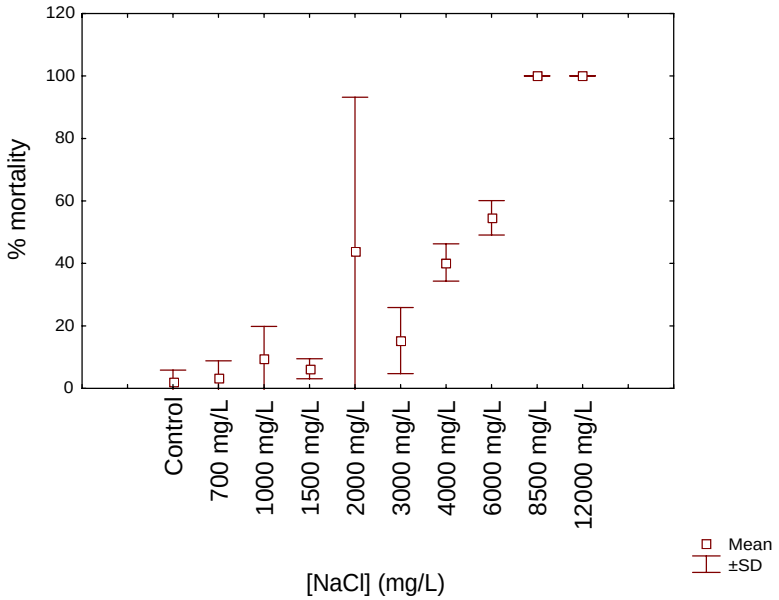


Figure 2.11 Means and standard deviations for mortality data of a short-term chronic toxicity test (10 days) where *E.elegans* was exposed to NaCl.

The square root transformed data gave the highest degree of normality (W = 0.89), but a Levene's test still indicated that the transformed data had unequal variance. A parametric ANOVA indicated that there was a significant difference among treatments ($p < 0,05$) and a parametric Tukey test indicated that the 6000 mg/L treatment was significantly different from the control ($p < 0,05$). A non-parametric Kruskal-Wallis ANOVA by ranks found no significant difference between any

treatments ($p < 0,05$). The 3000 mg/L treatment was taken to be the NOEC in this test, since using this treatment instead of the 4000 mg/L treatment would result in more conservative ACR transformations of the acute data and the standard deviations of this treatment did not overlap with those of the control (Figure 2.11). The 6000 mg/L treatment was likely indicated to be the LOEC by statistical testing because of the high intra-treatment variability in response of the 2000 mg/L treatment (Figure 2.11). Since the short-term chronic test used a re-circulating experimental design, only acute tests using the same experimental design and of 96 hours duration were considered. Three acute tests were identified with 96 hour LC_{50} values of 6898.7, 7625 and 8957 mg/L (Table 2.1). The ACR was calculated as the geometric mean of the LC_{50} values divided by the NOEC value of 3000 mg/L to get a result of 2.42.

Since no other chronic or short –term chronic data for Na_2SO_4 were available, the final ACR values chosen were:

NaCl: Geometric mean (2.9; 2.42) = 2.6.
Na_2SO_4: 3.17

All available 96 hour acute LC_{50} values were grouped by organism for NaCl and Na_2SO_4. The geometric means of the LC_{50}s for each organism were calculated for use in transformation to PNOEC values using the calculated ACRs and the results represented graphically in Figures 2.12 and 2.13 respectively. Source acute data (including species sample sizes) are shown in Table 2.1 and 2.2.

Due to the nature of the ACR extrapolation method, the relative sensitivity of macroinvertebrates according to the predicted chronic data will mirror that of the source acute data (Figures 2.12 and 2.13, Table 2.1 and 2.2). Table 2.4 lists the results of the ACR conversions of acute data for Na_2SO_4 and NaCl. Generally the most sensitive macroinvertebrates to both NaCl and Na_2SO_4 according to the source acute data and the ACR converted data are *Baetis harrisoni* (Ephemeroptera: Baetidae), *T.discolor* and *Tricorythus tinctus* (Ephemeroptera: Tricorythidae), *Burnupia stenochorias* (Gastropoda: Ancylidae), *Cloeon virgiliae* (Ephemeroptera: Baetidae), and *Oligoneuriopsis lawrencei* (Ephemeroptera: Oligoneuriidae).

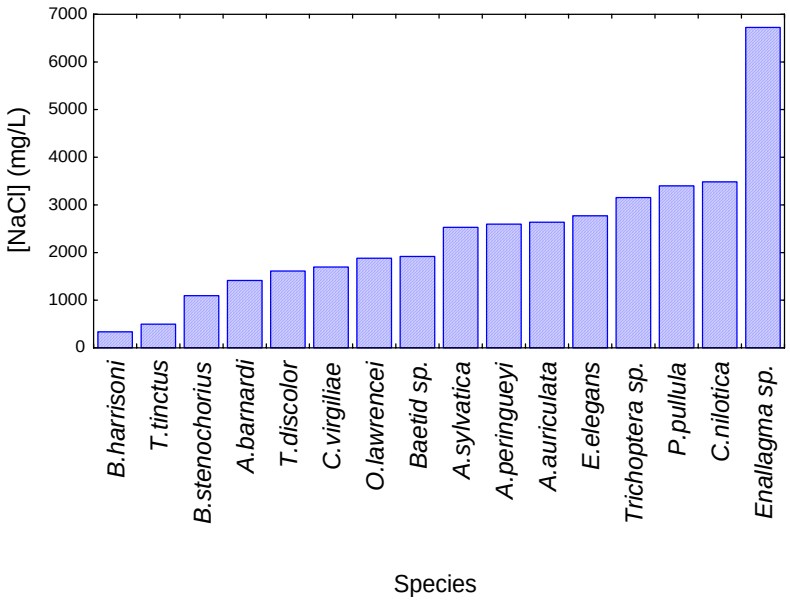


Figure 2.12 Graph showing comparative sensitivity as PNOEC (mg/L) values of South African macro-invertebrates contained in the IWR/UCEWQ toxicity database to NaCl according to ACR generated chronic tolerance data.

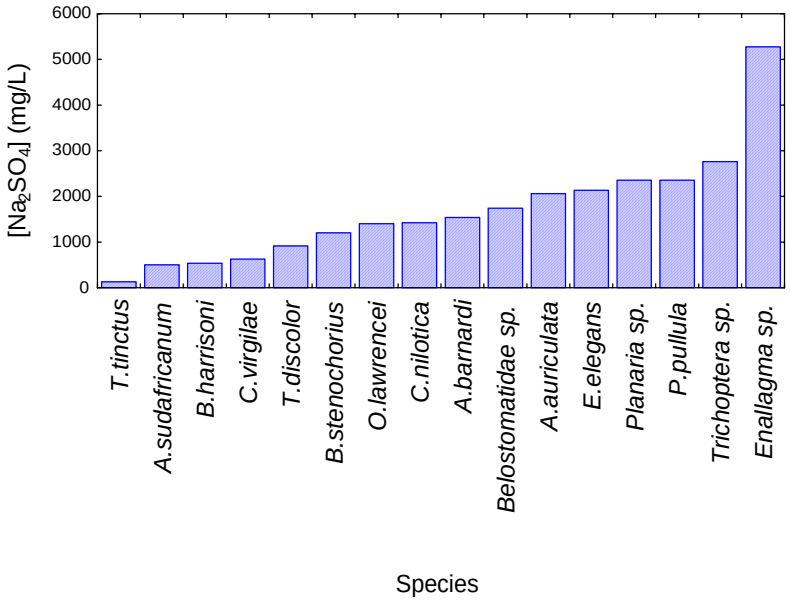


Figure 2.13 Graph showing comparative sensitivity as PNOEC (mg/L) values of South African macro-invertebrates contained in the IWR/UCEWQ toxicity database to Na_2SO_4 according to ACR generated chronic tolerance data.

The unidentified baetid (Ephemeroptera: Baetidae) species is sensitive to NaCl and *Afroptilum sudafricanum* (Ephemeroptera: Baetidae) is sensitive to Na_2SO_4. *Afronurus barnardi* (Ephemeroptera: Heptageniidae) is relatively sensitive to both Na_2SO_4 and NaCl. *C.nilotica* appears to be relatively tolerant of NaCl and relatively sensitive to Na_2SO_4. The macroinvertebrates that are relatively tolerant to both NaCl and Na_2SO_4 are *Adenophlebia auriculata* (Ephemeroptera: Leptophlebiidae) and *E.elegans*. *Plea pullula* (Hemiptera: Pleidae), the unidentified caddisfly (Trichoptera) species and the unidentified damselfly (*Enallagma*) species are particularly tolerant to both NaCl and Na_2SO_4. *Adenophlebia sylvatica* (Ephemeroptera: Leptophlebiidae) and *Afronurus peringueyi* (Ephemeroptera: Heptageniidae) were relatively tolerant to NaCl while the unidentified giant water bug (Hemiptera: Belostomatidae) species and unidentified flatworm (Platyhelminthes) are relatively tolerant to Na_2SO_4. Interestingly, the macroinvertebrates found in standing waters, namely the *Enallagma* (damselfly) species, the caddisfly species, *P.pullula*, and the Belostomatidae species were particularly tolerant to both salts. *C.virgiliae*, another lentic species, is however not particularly tolerant to either salt.

2.3.2 Extrapolations using Two - Step Linear Regression.

The results of LRA are listed in Table 2.3 and Table 2.4. More information about the acute data used (number of experiments and observations) is depicted in Table 2.8. A feature of extrapolating low LC_x values in the range of $LC_{0,01}$ to LC_{10} using LRA, is that the results for many species are negative (<0), and are therefore considered inappropriate for reporting. The extrapolation of LC_{50} chronic data yielded many more positive (>0) and therefore usable results. The relative inter-species sensitivity according to the extrapolated chronic data is reflected in Figures 2.14 – 2.17. Since Mayer *et al*. (1994) recommends associating the $LC_{0,01}$ - LC_{10} values to possible PNOECs, this study reports on the LC_1 values in inter-species and intra - species comparisons of extrapolated results since this is the modal value in the range of 0,01 - 10.

The relative sensitivity of species to NaCl according to extrapolated LC_1 values are reflected in Figure 2.14. *C.virgiliae* and *E.elegans* are shown to be particularly sensitive to NaCl. The caddisfly species, *C.nilotica*, *A.auriculata* and *B.stenochorias*

show relatively intermediate tolerance. The *Enallagma* (damselfly) species is reflected to have relatively high tolerance to NaCl.

The relative sensitivity of species to Na_2SO_4 according to extrapolated LC_1 values are reflected in Figure 2.16. The *Belostomatidae* species, *C.virgiliae* and *A.sudafricanum* are sensitive to Na_2SO_4. The caddisfly species and *A.auriculata* show intermediate tolerance. One again, the *Enallagma* (damselfly) species shows relatively high tolerance to Na_2SO_4.

The results of extrapolating LC_{50} values and applying a safety factor of 5 are generally more conservative than the extrapolated LC_1 values for both salts. The relative sensitivity of species to NaCl according to extrapolated LC_{50} values are reflected in Figure 2.15. *A.barnardi*, *A.sylvatica*, *A.auriculata*, *E.elegans*, the baetid species and *A.peringueyi* all show relatively low tolerance to NaCl. *T.discolor*, *C.virgiliae* and *B.stenochorias* show relatively intermediate tolerance and the caddisfly species, *C.nilotica* and the *Enallagma* (damselfly) species show relatively high tolerances to NaCl. The relative sensitivity of species to Na_2SO_4 according to extrapolated LC_{50} values are reflected in Figure 2.17. *O.lawrencei*, *T.discolor*, *B.stenochorias*, *A.sudafricanum*, *C.virgiliae*, *C.nilotica* and the caddisfly species all show relatively low tolerance to Na_2SO_4. The flatworm species, *A.auriculata* and *P.pullula* show relatively intermediate tolerance while the *Enallagma* (damselfly) species shows relatively high tolerance to Na_2SO_4.

The macroinvertebrates found in lentic systems i.e. the Enallagma (damselfly) species, the Belostomatidae species, *P.pullula* and the Caddisfly species show relatively higher tolerance to salts.

Table 2.3 A summary of the LC_x values obtained using Two-Step Linear Regression Analysis (LRA) (Mayer *et al*., 1994) and the associated R^2 values for Na_2SO_4.

Species	**$LC_{0,01}$**		**$LC_{0,1}$**		**LC_1**		**LC_5**		**LC_{10}**		**LC_{50}**	
	mg/L	**R^2**	**mg/L**	**R^2**	**mg/L**	**R^2**	**mg/L**	**R^2**	**mg/L**	**R^2**	**mg/L**	**R^2**
A.auriculata	1169	0,09	1026	0,12	3497	0,16	4042	0,21	4632	0,25	9027	0,34
A.barnardi	< 0		< 0		< 0		< 0		851	0,95	< 0	
A.sudafricanum	167	0,50	458	0,48	907	0,47	1469	0,46	1876	0,40	1469	0,46
B.stenochorias	411	0,07	31	0,23	< 0	0,41	31	0,47	3046	0,54	1270	0,65
Belastomatidae sp.	< 0		< 0		213		1694		< 0		5036	0,89
C.nilotica	< 0		< 0		< 0		< 0		< 0		3817	0,67
C.virgiliae	936	0,09	936	0,21	577	0,36	673	0,47	937	0,52	2871	0,83
Enallagma sp.	6729	0,02	6292	0,05	6895	0,06	9379	0,04	11151	0,03	16826	0,03
O.lawrencei	< 0		< 0		< 0		< 0		< 0		234	0,86
P.pullula	< 0		< 0		< 0		< 0		< 0		9133	0,82
Planaria sp.	< 0		< 0		< 0		319	0,92	653	0,96	7042	0,91
T.discolor	< 0		< 0		< 0		< 0		< 0		1219	0,25
Trichoptera sp.	1375	0,07	1543	0,05	2193	0,03	3200	0,04	4043	0,09	4859	0,83

Table 2.4 A summary of the LC_x values obtained using Two-Step Linear Regression Analysis (LRA) (Mayer et al., 1994) and the associated R^2 values for NaCl

Species	**$LC_{0,01}$**		**$LC_{0,1}$**		**LC_1**		**LC_5**		**LC_{10}**		**LC_{50}**	
	mg/L	**R^2**	**mg/L**	**R^2**	**mg/L**	**R^2**	**mg/L**	**R^2**	**mg/L**	**R^2**	**mg/L**	**R^2**
A.auriculata	1970	0,89	1970	0,89	2087	0,89	2209	0,90	23	0,92	1130	0,97
A.barnardi	515	0,20	< 0		< 0		101	0,15	224	0,26	575	0,62
A.peringueyi	< 0		< 0		< 0		< 0		< 0		1424	0,66
A.sylvatica	< 0		< 0		< 0		< 0		< 0		625	0,81
B.stenochorias	2835	0,14	2684	0,01	2611	0,02	1601	0,25	1168	0,57	3699	0,78
Baetid sp.	< 0		< 0		< 0		413	0,03	679	0,05	1391	0,71
C.nilotica	548	0,61	943	0,69	1599	0,75	2659	0,77	3511	0,76	8999	0,52
C.virgiliae	502	0,83	502	0,83	502	0,83	< 0		< 0		3403	0,95
E.elegans	64	0,55	233	0,54	560	0,52	1089	0,53	1543	0,57	1288	0,27
Enallagma sp.	7699	0,38	7283	0,69	7055	0,85	7521	0,91	8003	0,92	10127	0,97
T.discolor	< 0		< 0		< 0		< 0		353	0,65	2428	0,36
Trichoptera sp.	596	0,85	855	0,78	1292	0,75	2117	0,83	2940	0,87	8586	0,78

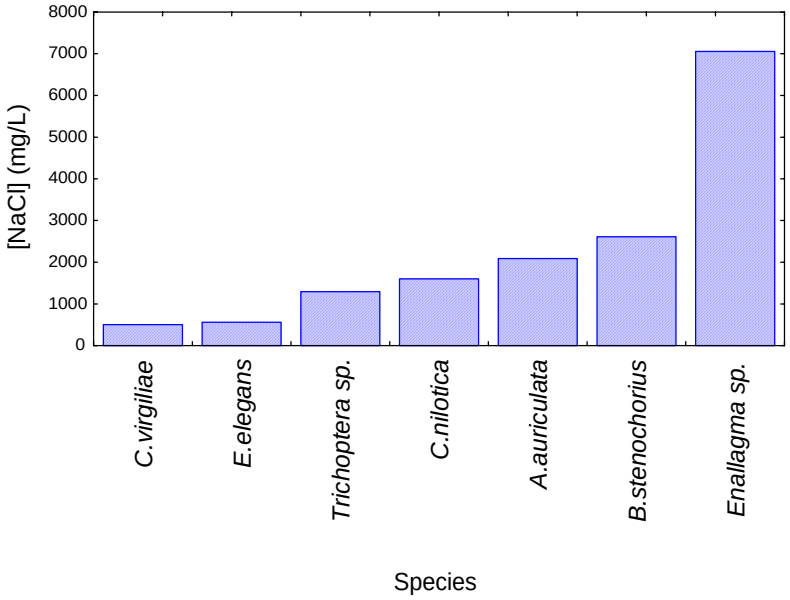


Figure 2.14 Graph showing comparative sensitivity of macro-invertebrates contained on the IWR/UCEWQ toxicity database to NaCl according to chronic tolerance data generated by the Two – Step Linear Regression Analysis Method (LRA) (Mayer et al., 1994) using the asymptotic LC1 value.

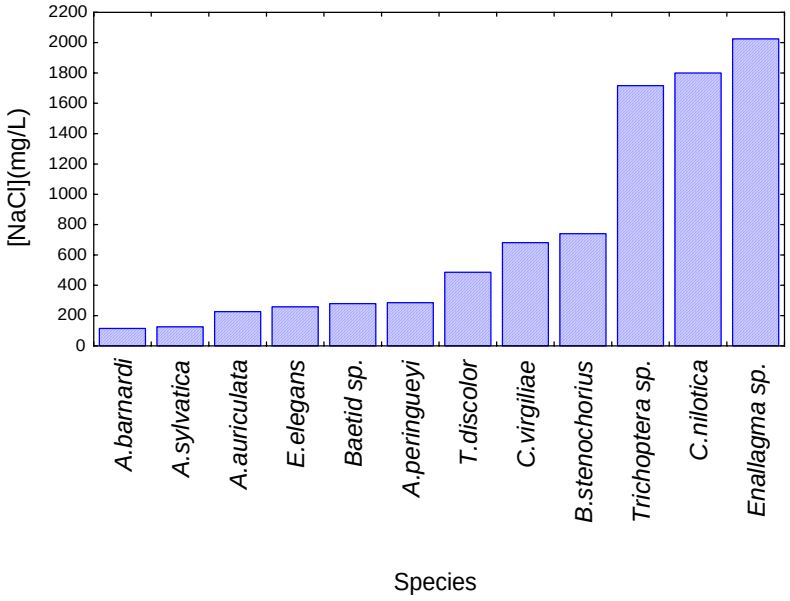


Figure 2.15 Graph showing comparative sensitivity of macro-invertebrates contained on the IWR/UCEWQ toxicity database to NaCl according to chronic tolerance data generated by the Two – Step Linear Regression Analysis Method (LRA) (Mayer *et al.*, 1994) using the asymptotic LC_{50} value /5.

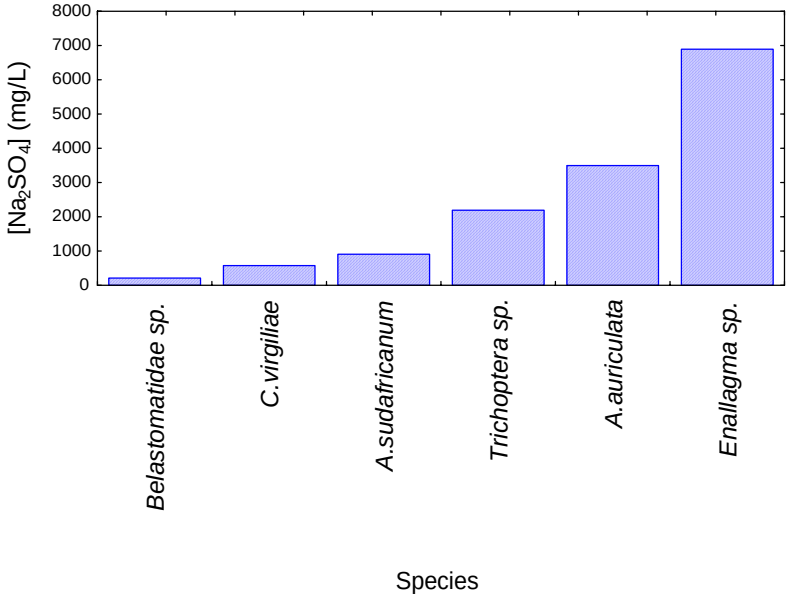


Figure 2.16 Graph showing comparative sensitivity of macro-invertebrates contained on the IWR/UCEWQ toxicity database to Na_2SO_4 according to chronic tolerance data generated by the Two –Step Linear Regression Analysis Method (LRA) using the asymptotic LC_1 value.

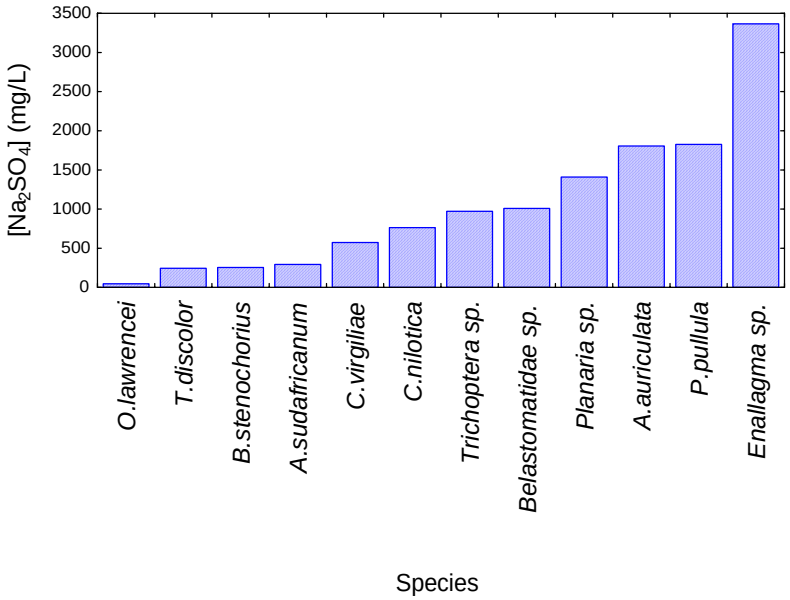


Figure 2.17 Graph showing comparative sensitivity of macro-invertebrates contained on the IWR/UCEWQ toxicity database to Na_2SO_4 according to chronic tolerance data generated by the Two – Step Linear Regression Analysis Method (LRA) (Mayer *et al*., 1994) using the asymptotic LC_{50} value /5.

2.3.3 Extrapolations using Multi-Factor Probit Analysis

Figure 2.18 and 2.19 illustrate how threshold times were calculated for macroinvertebrates exposed to Na_2SO_4 and NaCl respectively. A threshold time of 100 hours was calculated for *A.barnardi*, *C.virgiliae*, *E.elegans* and *P.pullula* exposed to Na_2SO_4 (Figure 2.18). A threshold time of 100 hours and 60 hours was calculated for *B.harrisoni* and *C.virgiliae* exposed to NaCl respectively (Figure 2.19). It was found in initial experiments to design a chronic test protocol that mortality for *C.nilotica* exposed to NaCl stabilises within 15 days (see Chapter 3) so a threshold time of 336 hours was chosen for *C.nilotica* exposed to NaCl. The process of determining threshold times is necessary to establish at what exposure times different species achieve a stabilisation of mortality when exposed to toxicants.

Table 2.5 and Table 2.6 list the results of extrapolations using MPA for Na_2SO_4 and NaCl respectively. More information about the acute data used (number of experiments and observations) is depicted in Table 2.8. Lee *et al*. (1995) recommend associating low LC_x values in the range of $LC_{0,01} - LC_{10}$ with possible PNOEC values. This study reports on LC_1 values for comparison of extrapolated inter and intra species chronic data. This method also tended to produce negative results (<0) when extrapolating low LC_x values. The extrapolation of LC_{50} values followed by the application of a safety factor of 5 produced many more usable data.

Model A2 produced the best - fit 70 % of the time. Although extrapolated low effects ($LC_{0,01} - LC_{10}$) appear to be much more conservative than those produced by LRA, LC_{50} values produced using MPA appear to be unrealistically high in many cases (Table 2.4). The extrapolated LC_{50} for *E.elegans* exposed to NaCl for example was close to 50 000 mg/L.

The results of extrapolated LC_1 values for NaCl are reflected in Figure 2.20. *A.sylvatica, E.elegans* and the *Baetid sp.* appear to be the most sensitive of the species tested to NaCl. *C.nilotica*, the *Enallagma sp.* (damselfly) and *C.virgiliae* appear to have intermediate tolerance. *A.auriculata* is reflected as having a relatively high tolerance to NaCl.

The results of extrapolated LC_1 values for Na_2SO_4 are reflected in Figure 2.22. *B.stenochorias*, the *Planaria sp*. and *C.virgiliae* are shown to be relatively sensitive. The caddisfly species, *A.auriculata* and the *Belostomatidae sp.* are shown to have intermediate tolerance while the *Enallagma* (damselfly) species is shown to have a relatively high tolerance to Na_2SO_4.

The results of extrapolated LC_{50} values followed by the application of a safety factor of 5 for NaCl are reflected in Figure 2.21. *T.tinctus*, *B.harrisoni*, *C.nilotica*, the Baetid species, *T.discolor*, *P.pullula*, *C.virgiliae*, *A.barnardi* and *A.peringueyi* all appear to be sensitive to NaCl. *A.auriculata*, the *Enallagma* (damselfly) species and *A.sylvativa* show intermediate tolerance while *E.elegans* shows an unrealistically high tolerance.

The results of extrapolated LC_{50} values followed by the application of a safety factor of 5 for Na_2SO_4 are reflected in Figure 2.23. *T.discolor*, *B.harrisoni*, *C.nilotica*, the Flatworm species and the Belostamatidae species all show relatively low tolerance. The *Enallagma* (damselfly) species, *C.virgiliae*, *A.auriculata*, *B.stenochorias* and the caddifly species show relatively intermediate tolerance while *A.barnardi*, *P.pullula* and *E.elegans* show relatively high tolerance to Na_2SO_4.

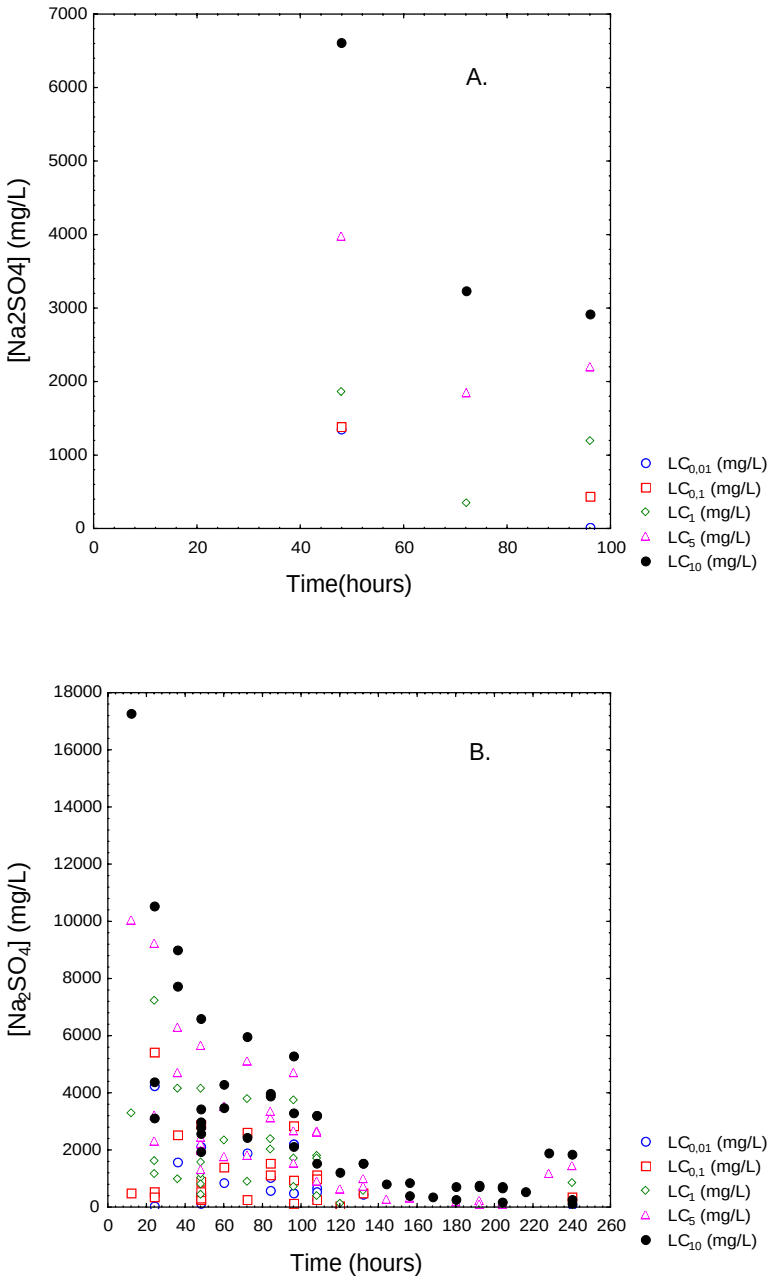


Figure 2.18 $LC_{0,01}$ – LC_{10} values were plotted against time for various macro-invertebrates exposed to Na_2SO_4. Time taken for mortality to stabilise was taken to be the threshold time. A - *A.barnardi*; B - *C.nilotica*; C - *C.virgiliae*; D - *E.elegans*; E - *P.pullula*; F - *T.discolor*. (Note: different scales of y-axis are a result of the variable sensitivities of the different species).

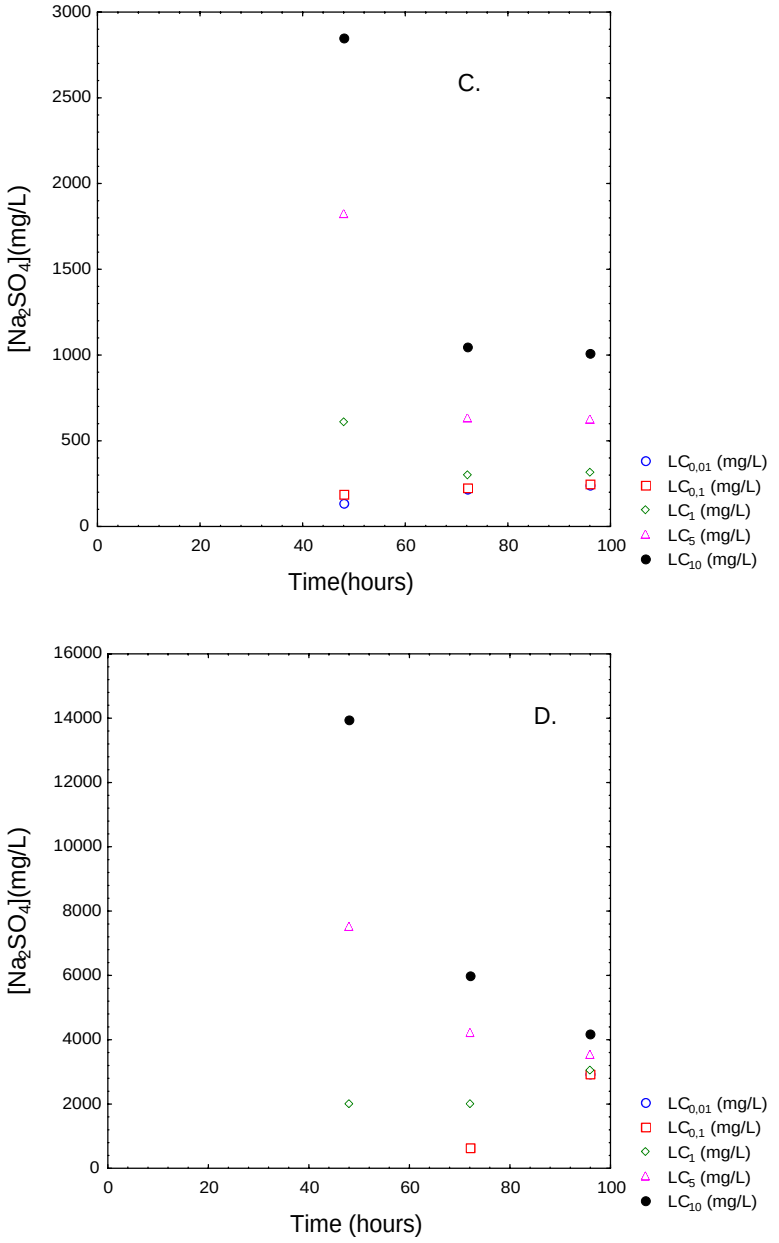


Figure 2.18 continued. $LC_{0,01}$ – LC_{10} values were plotted against time for various macro-invertebrates exposed to Na_2SO_4. Time taken for mortality to stabilise was taken to be the threshold time. A - *A.barnardi*; B - *C.nilotica*; C - *C.virgiliae*; D - *E.elegans*; E - *P.pullula*; F - *T.discolor*. (Note: different scales of y-axis are a result of the variable sensitivities of the different species).

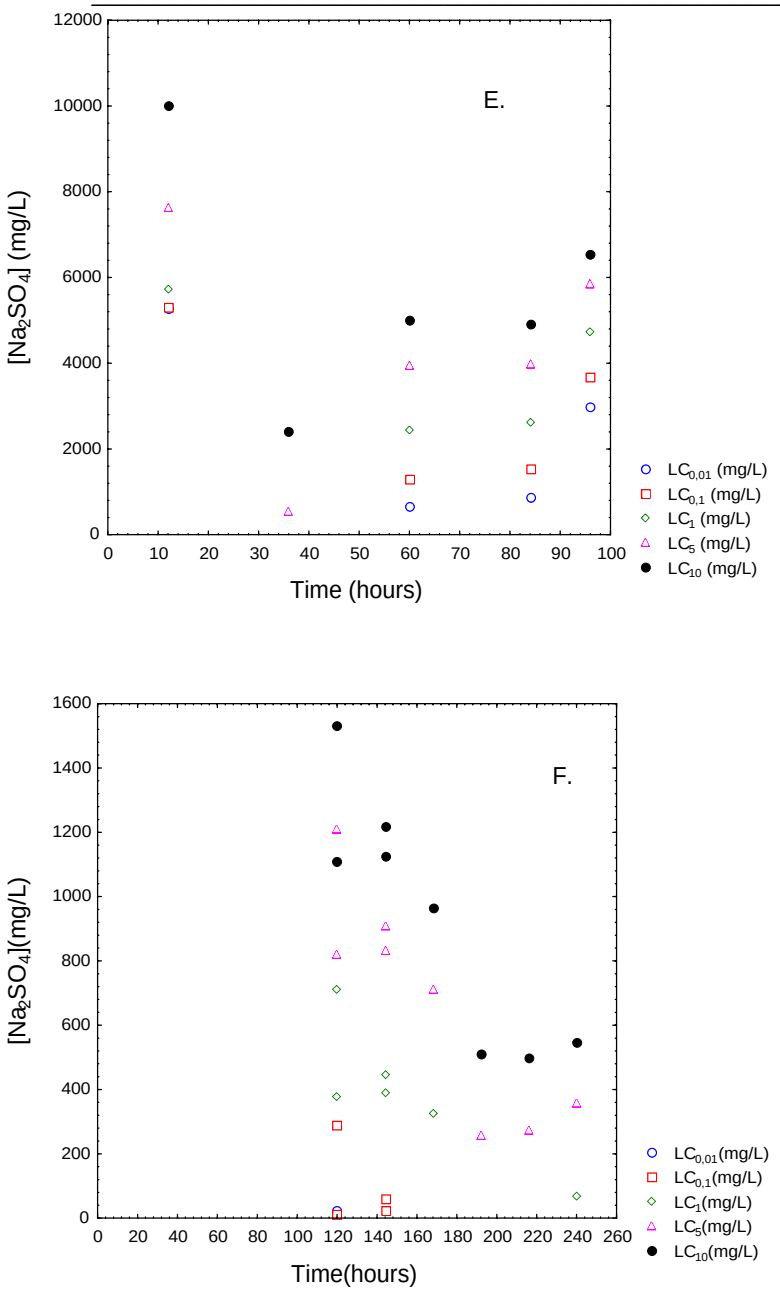


Figure 2.18 continued. $LC_{0,01}$ – LC_{10} values were plotted against time for various macro-invertebrates exposed to Na_2SO_4. Time taken for mortality to stabilise was taken to be the threshold time. A - *A.barnardi*; B - *C.nilotica*; C - *C.virgiliae*; D - *E.elegans*; E - *P.pullula*; F - *T.discolor*. (Note: different scales of y-axis are a result of the variable sensitivities of the different species).

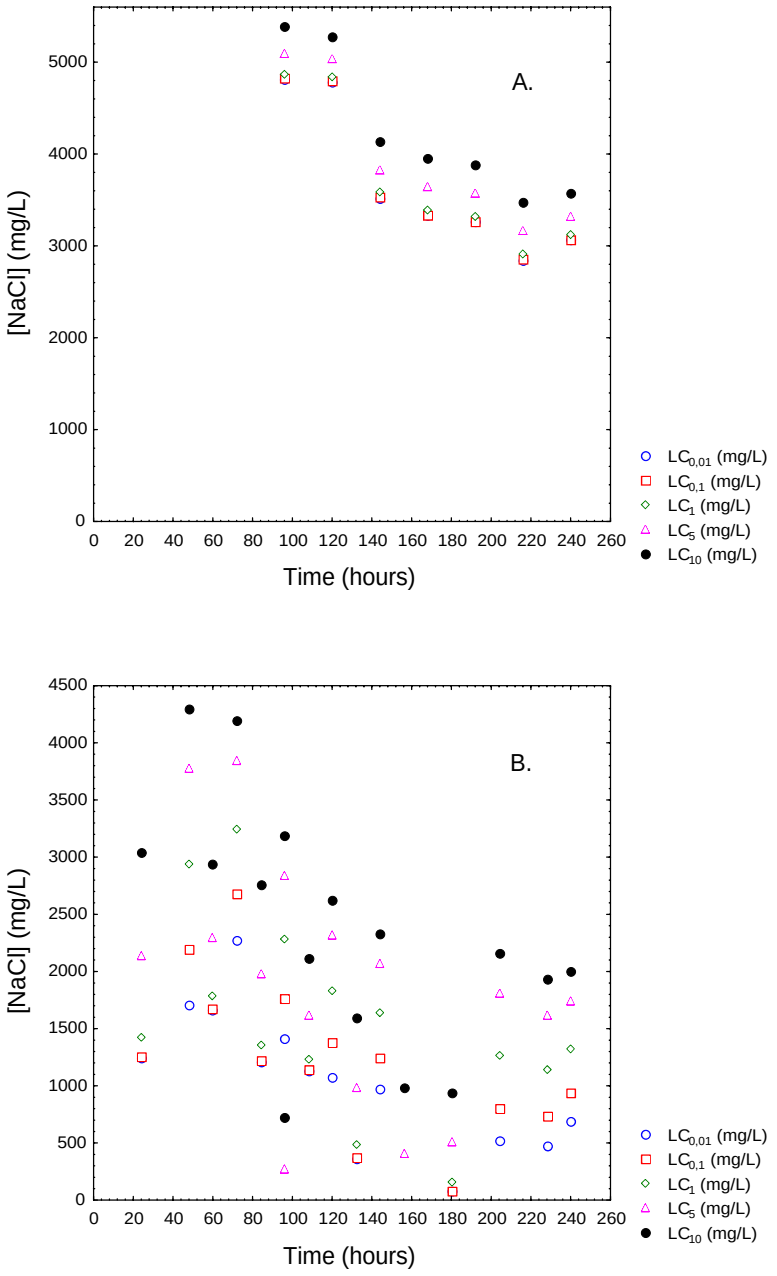


Figure 2.19 $LC_{0,01}$ – LC_{10} values were plotted against time for various macro-invertebrates exposed to NaCl. Time taken for mortality to stabilise was taken to be the threshold time. A – *A.auriculata*; B – A.*barnardi*; C – *B.harrisoni*. (Note: different scales of y-axis are a result of the variable sensitivities of the different species).

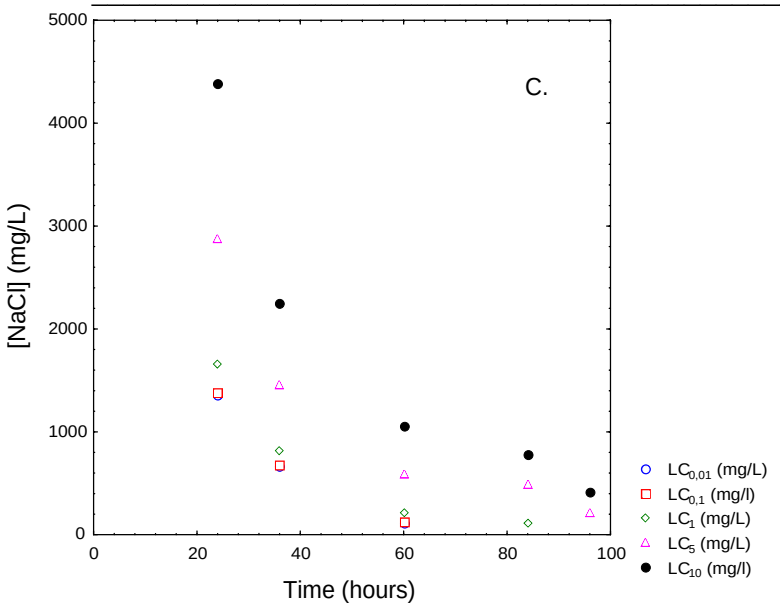


Figure 2.19 continued. $LC_{0,01}$ – LC_{10} values were plotted against time for various macro-invertebrates exposed to NaCl. Time taken for mortality to stabilise was taken to be the threshold time. A – *A.auriculata*; B – A.*barnardi*; C – *B.harrisoni*. (Note: different scales of y-axis are a result of the variable sensitivities of the different species).

Table 2.5 A summary of the LC_x values obtained using MPA (Lee *at al*., 1995) for Na_2SO_4 associated with goodness of fit values.

Organism	**Time to Threshold (hours)**	**Model**	**Ch^2 goodness of fit**	**Tabulated chi ^{2}**	**$LC_{0,01}$ (mg/L)**	**$LC_{0,1}$ (mg/L)**	**LC_1 (mg/L)**	**LC_5 (mg/L)**	**LC_{10} (mg/L)**	**LC_{50} (mg/L)**
A.auriculata	240	C2	216	259	< 0	24	943	2462	3717	13104
A.barnardi	100	A2	22	34	< 0	< 0	< 0	94	1098	20633
B.harrisoni	240	A2	15	53	< 0	< 0	< 0	66	265	1331
B.stenochorias	240	B2	104	132	< 0	< 0	516	2075	3496	16264
Belostomatidae sp.	240	C2	28	70	< 0	412	1186	2235	2990	7378
C.nilotica	240	A2	274	761	< 0	< 0	< 0	476	841	3037
C.virgiliae	100	A2	90	135	< 0	55	884	2177	3204	10324
E.elegans	100	A2	40	115	< 0	65	1468	4266	6901	32180
Enallagma sp.	240	A2	104	138	137	662	1646	3016	4019	10040
P.pullula	100	A2	55	95	< 0	< 0	< 0	701	2127	26377
Planaria sp.	240	B2	39	79	< 0	58	722	1671	2379	6768
T.discolor	240	A2	60	228	< 0	< 0	< 0	< 0	< 0	283
Trichoptera sp.	240	A2	174	181	< 0	92	1204	3149	4822	18301

Table 2.6 A summary of the LC_x values obtained using MPA (Lee *at al*., 1995) for NaCl associated with goodness of fit values.

Organism	**Time to Threshold (hours)**	**Model**	**Ch^2 goodness of fit**	**Tabulated chi ^{2}**	**$LC_{0,01}$ (mg/L)**	**$LC_{0,1}$ (mg/L)**	**LC_1 (mg/L)**	**LC_5 (mg/L)**	**LC_{10} (mg/L)**	**LC_{50} (mg/L)**
A.auriculata	240	A2	79	131	195	606	1440	2700	3677	10190
A.barnardi	240	B2	123	226	< 0	< 0	< 0	< 0	< 0	6928
A.peringueyi	240	A2	265	406	< 0	< 0	< 0	< 0	237	8243
A.sylvatica	240	B2	104	162	< 0	< 0	165	1225	2360	16514
B.harrisoni	100	A2	24	80	< 0	< 0	< 0	161	356	1623
Baetid sp.	240	A2	182	271	< 0	< 0	187	719	1155	4350
C.nilotica	336	A2	181	254	< 0	175	571	1115	1509	3838
C.virgiliae	60	B2	38	79	17	307	882	1730	2377	6563
E.elegans	240	C2	1061	1163	< 0	< 0	182	1895	4169	48501
Enallagma sp.	240	A2	128	145	< 0	182	878	2076	3093	11115
P.pullula	240	A2	65	106	< 0	< 0	< 0	< 0	376	6121
T.discolor	140	A2	524	1032	< 0	< 0	< 0	101	513	5806
T.tinctus	140	A2	51	166	< 0	< 0	< 0	< 0	< 0	860

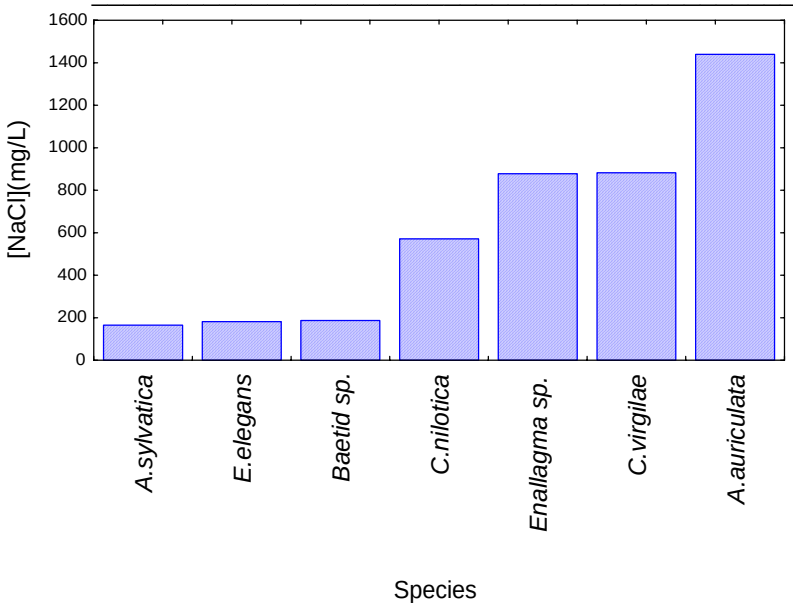


Figure 2.20 Graph showing comparative sensitivity of macro-invertebrates contained on the IWR/UCEWQ toxicity database to NaCl according to chronic tolerance data generated by the Multi-Factor Probit Analysis Method (MPA) (Lee *at al*., 1995) using the LC_1 value.

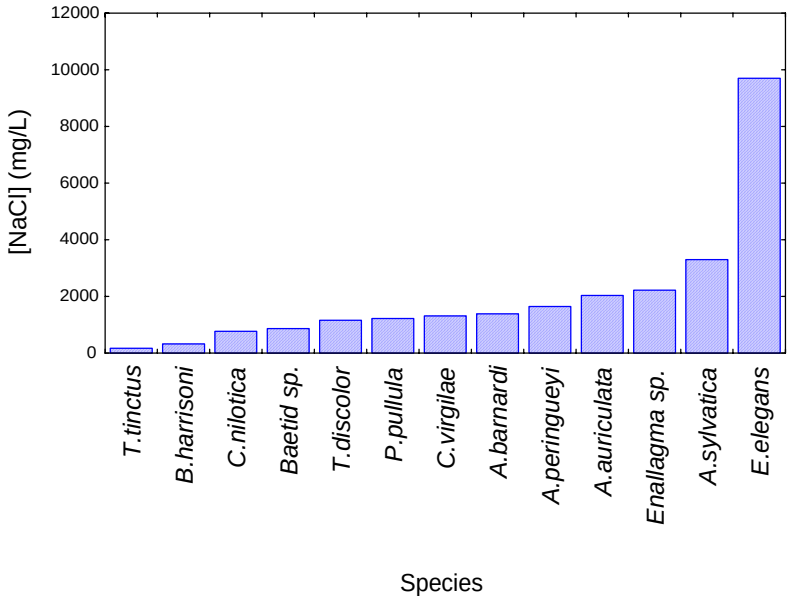


Figure 2.21 Graph showing comparative sensitivity of macro-invertebrates contained on the IWR/UCEWQ toxicity database to NaCl according to chronic tolerance data generated by the Multi-Factor Probit Analysis Method (MPA) (Lee *et al*., 1995) using the LC_{50} value / 5.

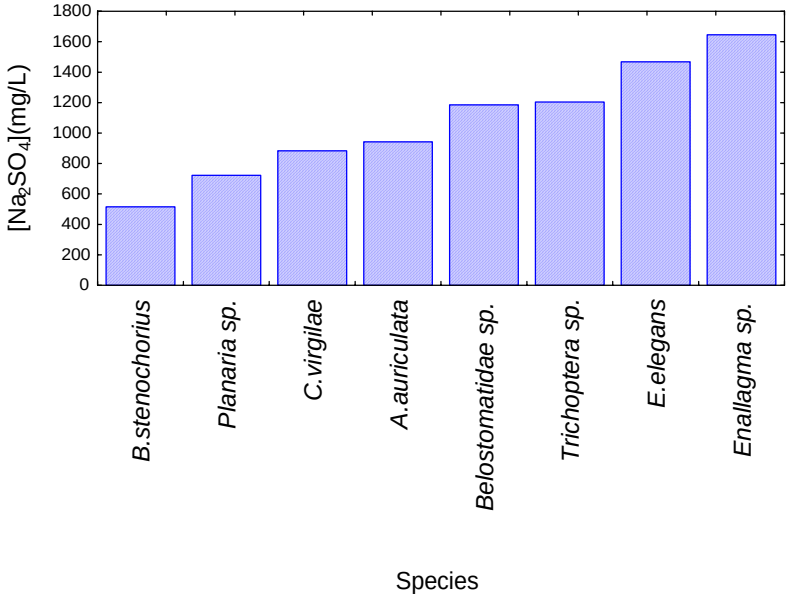


Figure 2.22 Graph showing comparative sensitivity of macro-invertebrates contained on the IWR/UCEWQ toxicity database to Na_2SO_4 according to chronic tolerance data generated by the Multi-Factor Probit Analysis Method (MPA) (Lee et al., 1995) using the LC_1 value.

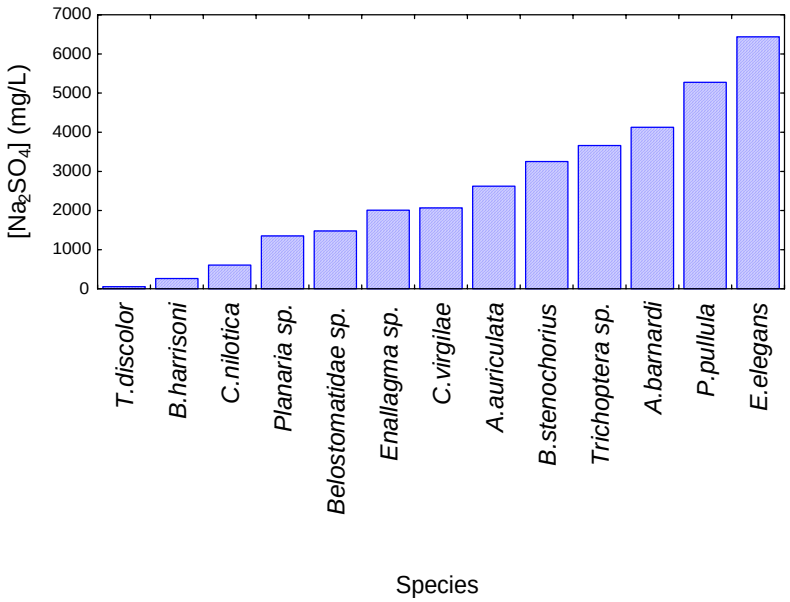


Figure 2.23 Graph showing comparative sensitivity of macro-invertebrates contained on the IWR/UCEWQ toxicity database to Na_2SO_4 according to chronic tolerance data generated by the Multi-Factor Probit Analysis Method (MPA) (Lee et al., 1995) using the LC_{50} value / 5.

2.3.4 Comparative protection offered by the various extrapolation methods

A summary of PNOEC values using the different extrapolation methods is shown in Table 2.7. Eleven comparisons are possible among the extrapolation techniques for both salts combined where all methods give a comparable value for a particular species. Chronic LC_1 values using MPA are the most conservative, while chronic LC_{50} values produced by LRA and divided by a safety factor of 5 are slightly less conservative. Chronic LC_1 values using LRA are about as conservative as chronic LC_{50} values divided by a safety factor of 5 produced by MPA. The least conservative PNOEC values are produced by the ACR method. The inter - species variability of tolerance to NaCl and Na_2SO_4 as expressed by all the extrapolated chronic data are shown in Figure 2.24 and Figure 2.25 respectively. Generally the median of all extrapolated PNOEC values for NaCl fall under 2000 mg/L except that of the *Enallagma* (damselfly) species. The variability in extrapolated PNOECs for Na_2SO_4 are higher than those for NaCl. Generally the extrapolated PNOECs are less than 3000 mg/L.

Table 2.7 A summary of extrapolated chronic data, for NaCl and Na_2SO_4, for each of the extrapolation techniques. (N/A reflects where there were insufficient data to undertake the necessary extrapolations).

	NaCl					**Na_2SO_4**				
Species	**ACR**	**LRA (LC_{50}/5)**	**MPA (LC_{50}/5)**	**LRA (LC_1)**	**MPA (LC_1)**	**ACR**	**LRA (LC_{50}/5)**	**MPA (LC_{50}/5)**	**LRA (LC_1)**	**MPA (LC_1)**
A.auriculata	2636	226	2038	2087	1440	2060	1805	2621	3497	943
A.barnardi	1413	115	1385	<0	<0	1540	<0	4127	<0	<0
A.peringueyi	2596	285	57	<0	<0	N/A	N/A	N/A	N/A	N/A
A.sudafricanum	N/A	N/A	N/A	N/A	N/A	500	294	<0	907	<0
A.sylvatica	2529	125	3304	<0	165	N/A	N/A	N/A	N/A	N/A
B.harrisoni	338	<0	325	<0	<0	537	<0	266	<0	<0
B.stenochorias	1093	740	<0	2611	<0	1202	254	3253		516
Baetid sp.	1919	278	870	23	187	N/A	N/A	N/A	N/A	N/A
Belostomatidae sp.	N/A	N/A	N/A	N/A	N/A	1743	1007	1476	213	1186
C.nilotica	3483	1800	768	1600	571	1424	763	607	<0	<0
C.virgiliae	1695	681	1313	502	882	627	574	2065	577	884
Trichoptera sp.	3154	1717	<0	1292	<0	2762	972	3660	2193	1204
E.elegans	2772	258	9700	560	182	2135	<0	6436	<0	1468
Enallagma sp	6726	2025	2223	7055	878	5275	3365	2008	6898	1646
O.lawrencei	1880	N/A	N/A	N/A	N/A	1402	47	<0	<0	<0
P.pullula	3399	<0	1224	<0	<0	2357	1827	5275	<0	<0
Planaria sp.	N/A	N/A	N/A	N/A	N/A	2355	1408	1354	<0	722
T.discolor	1611	486	1161	<0	<0	916	244	57	<0	<0
T.tinctus	497	<0	172	<0	<0	132	<0	<0	<0	<0

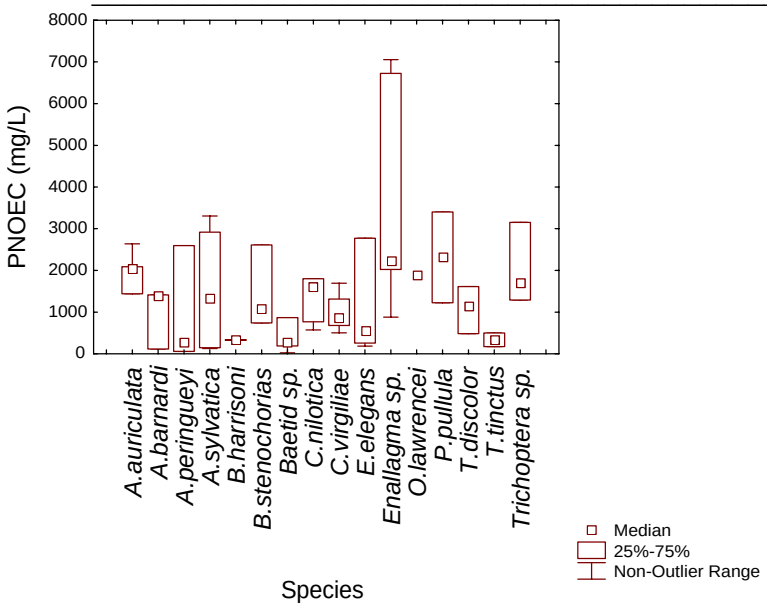


Figure 2.24 Graph showing the variability of inter species tolerance to NaCl chronic exposure according to chronic toxicity data generated by the ACR method, the LRA (LC_1) method, the LRA ($LC_{50}/5$) method, the MPA (LC_1) method and the MPA ($LC_{50}/5$) method.

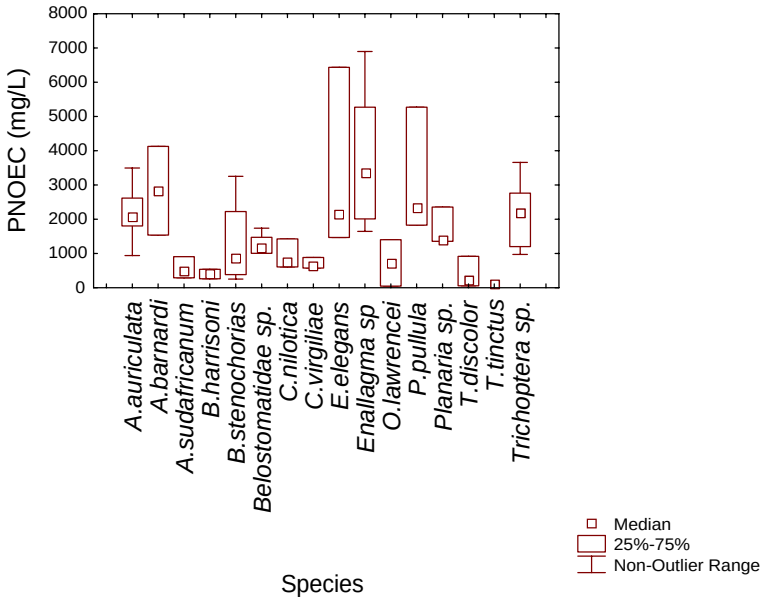


Figure 2.25 Graph showing the variability of inter species tolerance to Na_2SO_4 chronic exposure according to chronic toxicity data generated by the ACR method, the LRA (LC_1) method, the LRA ($LC_{50}/5$) method, the MPA (LC_1) method and the MPA ($LC_{50}/5$) method.

Table 2.8 Details of toxicological data used in the LRA (Mayer *et al*., 1994) and MPA (Lee *et al*., 1995) acute to chronic extrapolation methods. 'NA' - Not Applicable.

	NaCl			**Na2SO4**		
Species	**Number of Acute (96 hour) Experiments**	**Number of Short-Term Chronic (10 day) Experiments**	**Total Number of Mortality Observations**	**Number of Acute (96 hour) Experiments**	**Number of Short-Term Chronic (10 day) Experiments**	**Total Number of Mortality Observations**
A.auriculata	0	1	7	4	0	22
A.barnardi	0	2	16	1	0	3
A.peringueyi	1	3	28	NA	NA	NA
A.sudafricanum	NA	NA	NA	4	0	23
A.sylvatica	0	2	10	NA	NA	NA
B.harrisoni	2	0	5	1	0	3
B.stenochorias	1	1	7	2	0	10
Baetid sp.	1	2	13	NA	NA	NA
Belostomatidae sp.	NA	NA	NA	1	0	7
C.nilotica	5	0	16	1	3	33
C.virgiliae	1	0	7	2	0	10
Trichoptera sp.	3	0	8	3	0	6
E.elegans	3	11	55	2	0	6
Enallagma sp	3	0	6	3	0	8
O.lawrencei	NA	NA	NA	1	0	5
P.pullula	3	0	5	3	0	6
Planaria sp.	NA	NA	NA	0	1	9
T.discolor	0	13	34	1	3	11
T.tinctus	1	1	13	2	2	22

2.4 Discussion

The results of this study indicate that extrapolated LC_1 values using MPA and extrapolated LC_{50} values using LRA and subjected to a safety factor of 5 are the most conservative acute to chronic extrapolation methods. The conservativeness of predicted chronic responses generated by these extrapolation methods equates to greater protectiveness of the extrapolated values, as they are more likely to be equal or less than experimentally obtained chronic toxicity NOECs. The latter method is however preferable since there is a greater deal of confidence associated with an LC_{50} value (Clarke *et al*., 2002). The LC_{50} value is less model dependant than low effect values (Moore and Caux, 1997; Isnard *et al*., 2001). In addition, this method produced many more valid (> 0) data compared to the former method. The ACR method produced much less conservative predicted chronic data, and the protectiveness of the extrapolated data is doubtful. This however depends on the numerical value of the ACRs used. The ACR method does not take exposure period of acute and chronic toxicity tests into account, yet exposure time is a factor acting alongside toxicant concentration to determine toxicant affect on test organisms. The accuracy of MPA and LRA is increased with a greater input of short - term chronic data, since these methods incorporate the time of exposure in extrapolations.

The MPA and LRA methods were first suggested in 1995 and 1994 respectively (Lee *et al*., 1995; Mayer *et al*., 1994). The methods are based on sound, and easily demonstrated toxicological principles and are definitely preferable to application factors that have no scientific justification in their use. Yet there have been no subsequent published studies on the accuracy and usefulness of these methods. This may be a consequence of the lack of usable data obtained when extrapolating low effect levels ($LC_{0,01}$ – LC_{10}) using these methods, as many of the values obtained are less than zero. Most investigators of the methods are likely to disregard the methods totally because of this. Extrapolating an LC_{50} value and applying a safety factor is on one hand more scientifically defensible, as there is more confidence surrounding an LC_{50} value, yet the use of an application factor adds uncertainty to the extrapolations. Yet this method does produce usable data in the majority of cases. There is less uncertainty associated with using a safety factor of 5 on LC_{50} data, as compared to

using low effect LC_x values. A safety factor of 5 was applied to metal toxicants in the derivation of the ANZECC and ARMCANZ (2000) guidelines. The value of the safety factor was determined by the expert opinion of Dr John Chapman (NSW EPA) and was based on examining the data collated to derive the Trigger Values in the ANZECC and ARMCANZ (2000) guidelines (Warne, 2001). The safety factor was not applied to non-metal toxicity data as other toxicants had sufficient NOEC data (Warne, 2001). In a study of the relationship between EC_x and NOEC measures taken in chronic toxicity tests, Isnard *et al*. (2001) found that the median of the ratio of EC_x/NOEC for these tests was 2.3. Isnard *et al*. (2001) also found that on average, NOECs measured equated with an EC_x effect of 37% and in most cases there was not a big difference between EC_{50} and NOEC measures. While there was variation between regression models in low effect EC_x estimates, LC_{50} measures given by the models were very similar (Isnard *et al*., 2001). In view of the literature, it is suggested that a safety factor of 5 is protective in the majority of cases. This method is more scientifically rigorous than applying an ACR as the LRA estimation component of the method has sound theoretical basis. The LRA LC_{50} / 5 must however be validated by the results of experimental chronic toxicity data. This is addressed in Chapter 4.

A point of concern in acute to chronic toxicity data extrapolation is that most acute toxicity data available is likely to be those from toxicity tests performed on test organisms in older life stages. An experimental chronic test should ideally cover the duration of a test organism's entire lifecycle including more sensitive stages. Ideally therefore, acute data run on the most sensitive life stages of test animals should be used for acute to chronic mortality extrapolations. Much of the toxicity data contained on the IWR/UCEWQ database for aquatic insects and used in this study were derived from toxicity tests performed on juvenile stages, since adult stages are not aquatic. This includes all the mayfly, damselfly and caddisfly toxicity data. Acute toxicity data on the database for *C.nilotica* also included data from toxicity tests performed on very young juveniles (< 7 days). Therefore the majority of acute toxicity data used in acute to chronic extrapolation in this study (Table 2.1 and Table 2.2) were for sensitive lifecycle stages of the test organisms.

The issue of intra-species variability in terms of salinity tolerance may be of importance in acute to chronic mortality extrapolation. In these analyses, acute data were grouped by organism and toxicant, and no consideration was given to the source of the test organisms. Teschner (1995) found that *Daphnia magna* populations from brackish waters had a higher salinity tolerance than populations from fresher waters. Dalla Via (1987) found that the metabolic recovery of brackish water populations of *Palaemonetes antennarius* after exposure to high salinity was quicker than that of fresh water populations. The source of short-term chronic data in this study appeared to indicate that *E.elegans* populations in the Bushman's River are more tolerant to NaCl than populations in the Kat River. It can be argued however that disregarding region specific salinity tolerance of test organisms in acute to chronic extrapolations will produce generic salinity tolerance data for a particular species, after which site - specific information can be considered.

ACRs have been used in the compilation of the South African Water Quality Guidelines (DWAF, 1996) (Roux *et al*., 1996) and the ANZECC and ARMCANZ (2000) trigger values (Warne, 2001). The MPA and LRA acute to chronic extrapolation methods studied in this chapter provide alternatives to the ACR method to estimate chronic response from acute toxicity data. It is evident that chronic toxicity data estimated from LRA and MPA are more conservative and therefore more protective than that estimated from the use of ACRs. The LRA and MPA extrapolation techniques are easy to implement and are computationally inexpensive. Potentially they could produce valuable chronic tolerance data that could otherwise be difficult or too expensive to obtain. Chapter 4 deals with the accuracy of the LRA and MPA extrapolation methods in comparison with experimentally obtained chronic toxicity data. In addition, chapter 4 explores further the possible role of LRA and MPA in water quality guideline development.

Chapter 3: Chronic salt tolerance of *Caridina nilotica*

3.1 Introduction

Aquatic ecotoxicity tests form a vital role in hazard assessment in aquatic ecosystems (Rand, 1995). Although biomonitoring of an aquatic ecosystem can alert to a particular effect in an ecosystem, no indication of the causative agent is given (Fent, 2002). In addition, chemical analysis of water in an aquatic ecosystem can give an indication of the pollutants present, but will not indicate the toxicity of the pollutants to the organisms found in that aquatic ecosystem (Fent, 2002). Ecotoxicity tests therefore provide the link between the pollutants identified and the effects observed in the field (Palmer *et al*., 2002). The results of aquatic ecotoxicology tests are a resource for the generation of water quality guidelines (Roux *et al*., 1996; Warne, 2001). Chronic toxicity test data are particularly valuable for the generation of water quality guidelines. High reliability trigger values in the ANZECC and ARMCANZ (2000) trigger values were generated using single species chronic or multi-species toxicity data (Warne, 2001).

3.1.1 Definition and general description of chronic toxicity tests

Rand *et al.* (1995) give definitions for chronic and acute toxicity tests. Aquatic toxicology attempts to define a cause – effect relationship between aquatic organisms and a particular substance. Acute toxicity tests are typically of short duration and usually measure lethality (Rand *et al*., 1995). Chronic toxicity tests are usually run over a test organism's entire lifecycle, or at least a large proportion of the lifecycle, and sub-lethal endpoints such as reproduction or growth are measured (Rand *et al*., 1995). Warne (2001) gives more specific exposure time dependant definitions of acute and chronic toxicity tests that were used to compile the Australian and New Zealand Water Quality Guidelines for Toxicants (ANZECC and ARMCANZ 2000). Chronic toxicity tests for multi-celled organisms were defined as tests where the exposure duration was greater than 96 hours. Acute tests were defined as tests where exposure time was greater than or equal to 24 hours and less than or equal to 96 hours.

3.1.2 Statistical methods for analysing chronic toxicity test data

There are two main methods of analysing toxicity data. These are the LC_x regression approach and the NOEC/LOEC approach (Warne, 1998; Isnard *et al*., 2001). The NOEC is the highest concentration in a toxicity test that does not produce a statistically significant effect when compared to the control at $p < 0,05$ (Rand *et al*., 1995; Warne, 1998). The LOEC is the lowest concentration in a toxicity test that does produce a statistically significant effect when compared to the control at $p < 0,05$ (Rand *et al*., 1995; Warne, 1998). The LOEC and NOEC would be determined using hypothesis testing statistics, where the null hypothesis would be that the toxicant causes no significant effect as compared to the control (Chapman and Caldwell, 1996). LC_x values determined by regression are popular in the analysis of acute toxicity data but are not as frequently used with chronic toxicity data where the NOEC approach is predominantly used (Rand *et al*., 1995). Advantages and disadvantages associated with both approaches are discussed here.

The NOEC/LOEC is a computationally simple method with a straightforward experimental design (OECD, 1998). The main challenge is to select test concentrations that allow the most accurate identification of NOEC/LOEC values. The NOEC/LOEC approach has, however, frequently been criticised (Chapman and Caldwell, 1996; Moore and Caux, 1998; OECD, 1998; Warne, 1998; Crane and Newman, 2000; Isnard *et al*., 2001; Clarke *et al*., 2002):

1. The danger of false negatives i.e. because the ANOVA based design is essentially hypothesis testing where the null hypothesis is: *a given toxicant has no statistically significant response on a test subject compared to the control*, emphasis is placed in avoiding type I errors and the power (1 – Type II error) of the tests used to find NOECs are typically small (Chapman and Caldwell, 1996; Moore and Caux, 1997; OECD, 1998; Crane and Newman, 2000).
2. NOECs may be the highest concentration that have no statistically significant effect compared to the control but this does not mean that the estimated NOEC will not have a biologically significant effect (Moore and Caux, 1998; OECD, 1998; Warne, 1998; Crane and Newman, 2000).

3. There is not an optimal usage of data i.e. the only data used are the range from NOEC to LOEC, and consequently no subsequent extrapolation is possible (Moore and Caux, 1997; OECD, 1998; Clarke *et al*. 2002).
4. Choice of the multiple-comparison procedure (a statistical test) to determine which treatments are significantly different from the control after running an ANOVA and the statistical level of significance chosen has a large effect on the estimated LOEC (Chapman and Caldwell, 1996; Warne, 1998; Clarke *et al*. 2002).
5. The estimated LOEC and NOEC depend on the concentrations used in the test (Warne, 1998; Crane and Newman, 2000; Isnard *et al*., 2001; Clarke *et al*., 2002).
6. No indications of statistical confidence in the NOEC can be given with the NOEC estimated (Crane and Newman, 2000; Isnard *et al*., 2001; Clarke *et al*., 2002).
7. Poor statistical design in a regulatory context is rewarded, as the NOEC value is inevitably higher in an experiment with poor resolution, leading to more lenient regulations for the polluter (Moore and Caux, 1997; Isnard *et al*., 2001; Clarke *et al*., 2002).

Regression analysis is a common alternative approach and regression models such as Probit and Logit are commonly used (Rand *et al*., 1995). Usually in acute data, the LC measure taken is the LC_{50} measure as this measure has the most confidence (Rand *et al*., 1995) and is also relatively model independent (Moore and Caux, 1997). The advantages of regression analysis are:

1. Regression procedures allow the computation of confidence limits (Isnard *et al*. 2001).
2. Test statistics can inform whether a particular regression model adequately fits the toxicity test data (Moore and Caux, 1997).
3. All data are used (Moore and Caux, 1997).

The disadvantages are:

1. Confidence limits become excessively large at 5% and below (Moore and Caux, 1997; Clarke *et al*. 2002).

2. The concentration estimates in the lower LC_x ranges vary widely with the different models (Warne, 1998; Isnard *et al*, 2001).
3. Most common models follow the sigmoidal concentration response relationship. Data displaying hormesis will not fit these models well (Clarke *et al*., 2002).
4. The treatments must be chosen with the aim that toxicity results will be suitable for regression e.g. five or more treatments, treatments with partial effects and a treatment with 100 % mortality (Clarke *et al*., 2002).
5. Linear regression must be limited to interpolation situations because linear regression cannot predict toxicity outside the boundaries of the chosen treatments (Clarke *et al*., 2002).
6. Isnard *et al*. (2001) found that although goodness of data fit was related to the quality of the toxicity data, certain models performed better than others.

The high error associated with LC_x values can be reduced by estimating toxicity via interpolating between actual tested concentrations rather than extrapolating the effect of low concentrations (Warne, 1998). To achieve this, toxicity tests should have more treatments in the lower concentrations, larger numbers of replicates and more test specimens per concentration (Warne, 1998; Isnard *et al.*, 2001). Tests such as this would only be successful if the experimenter had a good idea of the range of tolerance of the test organism. Using this experimental design where a range finding test has not been done may result in an inconclusive concentration-response result.

Although there are many problems with the LOEC/NOEC approach, regulating agencies have not done much to standardise a viable alternative. Warne (1998) recommends that the use of the LOEC/NOEC approach in chronic toxicity testing be phased out as suitable LC_5 data from chronic testing becomes available. Suitable chronic data for regression analysis with the aim of determining an accurate LC_5 value, as mentioned before would have many treatments at lower concentrations, large numbers of replicates and many test organisms per concentration (Warne, 1998).

3.1.3 The degree of environmental realism of chronic data as compared to acute data

Persoone *et al.* (1990) cited a number of papers indicating that data on lethal concentrations of chemicals in short - term tests can accurately predict mortalities in the field. Nevertheless, acute data cannot be used to indicate safe, long - term exposure concentrations of a particular toxicant (Warne, 1998). Warne (1998) stresses the importance of chronic toxicity data for developing water quality guidelines. Water quality guidelines should protect organisms from long term exposure to toxicants (Warne, 1998). Chronic toxicity data are more indicative of the effects of long term toxicant exposure and are therefore more useful than acute toxicity data. Heming *et al*. (1989) showed that the organochlorine pesticide methoxychlor displayed a delayed toxic effect to certain fish species. Therefore an acute test may have underestimated the toxic effect of this particular pesticide.

3.1.4 Environmental realism of toxicity tests

There are concerns that ecotoxicological tests are too simplistic to reflect real life dose - response relationships since ecotoxicological tests have evolved more from the field of toxicology than ecology (Calow, 2003). Ecosystems are characterised by complex interactions between many species, however the majority of toxicity tests to date have been single species tests. The aim of using single species toxicity tests has been to standardise environmental factors so as to make experiments reproducible (Warne, 1998) but this has been to the expense of environmental realism (Cairns, 1983). A common theme in the literature is the need for toxicity tests that recognise a higher ecological level than just single species tests (Cairns, 1983). Results of single species toxicity tests run under constant conditions are assumed to reflect tolerance in field situations where many species co-exist and conditions are changing over time and space (Cairns, 1983). In reality, toxicity of a particular toxicant may be altered by other water quality parameters in the same environment (Cairns, 1983). For example, the toxicity of certain metals such as lead is affected by water hardness (Warne, 2001). Ecological characteristics such as ongoing functioning of ecosystems despite the loss of individual species, are not addressed in simple single species toxicity tests. A USEPA study found that single species tests were not a good predictor of effect of a

toxicant at the community level, although there was a relationship at a coarse level (Marcus and McDonald, 1992). Additionally, there have been claims that using water quality guidelines derived from single species tests have resulted in no disasters (Cairns, 1983).

Within single species toxicity testing, chronic toxicity tests will generally give more environmentally accurate tolerance information than acute toxicity tests. This is reflected by the ANZECC and ARMCANZ (2000) water quality guidelines which chose chronic toxicity data in preference to acute toxicity data to compile water quality guidelines (Warne, 2001). Organisms in the field are unlikely to be exposed to toxicants for only 96 hours or less and are more likely to be exposed for extended periods and may suffer sub-lethal affects such as inhibition of reproduction or growth. The lower concentration range and longer exposure time tested for in single species chronic toxicity tests therefore provide more environmentally realistic tolerance information. Multi –species toxicity test data using mesocosm or microcosm systems have been chosen in preference to single species chronic toxicity test data in the generation of the ANZECC and ARMCANZ (2000) water quality guidelines (Warne, 2001), indicating that the greatest deal of confidence of environmental reality is placed with this sort of toxicity testing. Unfortunately multi-species toxicity testing is complex and is still in a pioneering stage.

A common argument justifying the use of single species tests is that the use of apparently sensitive species will result in guidelines that will protect most of the species in a community (Cairns, 1983; Cairns 1986). This approach is based on the assumption that the biological response chosen will be the most sensitive possible, money and time saved from using a single species toxicity tests will be greater than expenses suffered due to rehabilitation of the environment after a bad management decision, and that the species chosen for sensitivity to a particular toxicant will show similar sensitivity to most other toxicants (Cairns, 1986). These assumptions are refuted by Cairns (1983, 1986), since only a few species are suitable for laboratory tests and they can hardly be termed the most sensitive species. Although it is reasonable to assume that safe levels of a particular toxicant for a sensitive organism will probably also be safe for most other organisms, it must first be proved that a particular test organism is markedly more sensitive to a particular toxicant than most

other organisms. It is unreasonable to assume that endpoints chosen for test species are the most sensitive endpoints possible. In fact, most endpoints in toxicity tests are chosen for reasons of replicability and ease of measurement (Cairns, 1986). In addition, a test species chosen for sensitivity from past results with other toxicants may not display the same sensitivity to the toxicant being studied. In a study of interspecies variability in sensitivity to toxicants, Sloof *et al.* (1983) concluded that it is not possible to predict the sensitivity of a particular species to one toxicant based on previous toxicity testing of the same species using a different toxicant. Other outcomes of the Sloof *et al*. (1983) study included the conclusion that the sensitivity of standard test organisms are not greater than the sensitivity of all other test organisms, indicating that safe levels of toxicants for standard test organisms will not necessarily protect all other biota. The results of single species toxicity tests may also be too sensitive as certain toxicants may not have the same degree of bio-availability in the field as they would in the laboratory (Cairns, 1983).

Although single species toxicity tests have obvious limitations, they remain a valuable method of determining safe toxicant concentrations for aquatic organisms and should be used until protocols for toxicity tests at a higher ecological level are developed such as multi-species tests using mesocosms and microcosms. It is however bad practice to extract an unreasonable amount of management information from single species tests (Cairns, 1986). New methods of guideline development, such as species sensitivity distributions, take the sensitivity of many organisms into account, and not just those that are most sensitive (Warne, 1998). Therefore, all tolerance information for a particular toxicant is valuable, regardless of the sensitivity of the test organism.

3.1.5 Sensitive early life stage tests

Many test species are not suitable for full lifecycle toxicity tests (Hutchinson *et al*., 1998). The preliminary study for this chapter for example, found that young *Caridina nilotica* juveniles have a high natural attrition rate and therefore full lifecycle tests cannot be preformed using this animal. Therefore partial lifecycle tests must be used to predict full lifecycle exposure results. Although literature exists on the relative sensitivity of different life stages in fish, there is a lack of knowledge on the relative sensitivity of life stages of aquatic invertebrates (Hutchinson *et al.*, 1998). This may be because many aquatic invertebrates have a short lifecycle and in many cases the

aquatic stage is only part of the lifecycle. However some literature does exist in this respect. Hutchinson *et al*. (1998) found that according to available chronic data on the ECETOC database, the sensitivity of invertebrate juveniles is greater than or equal to the sensitivity of invertebrate adults in 91 % of cases. McHohan *et al*. (1989) found that younger instars of the larvae of a caddisfly species were less tolerant than the older instars to cadmium. Kefford *et al.* (2004a) showed that the young juvenile stages of a species of stonefly and a species of caddisfly from the Barwon River in Australia are more sensitive to sea salt than older juvenile stages. Short - term chronic tests run on sensitive early life stages of a test organism may give an indication of lifecycle tolerance levels (McKim, 1977; Giedy and Graney, 1989). McKim (1977) demonstrated that the tolerance of early juvenile stages of certain fish species were comparable to those of full lifecycle tests within a factor of two. It has been suggested that individuals in a younger life stage would have a greater surface area to volume ratio thereby possibly making them more vulnerable to a toxin (Kefford *et al*., 2004a).

3.1.6 Field validation of toxicity test results

Tolerance data obtained from toxicity tests could be validated by field tolerance observations. This sort of correlation is however flawed as stated by Kefford *et al.* (2004b). The occurrence of an aquatic organism at a measured field salinity level may not indicate the maximum salinity level that that organism can tolerate. The organism's distribution may be limited to areas below a particular salinity level for reasons other than salinity. In addition, an organism may spend more sensitive life stages in a lower salinity level and may tolerate higher salinity in more mature stages. An individual of a particular species may also drift into a section of river from upstream and not be resident. Despite all these complications, Kefford *et al.* (2004b) found a correlation between macroinvertebrate LC_{50}s and maximum toxicant concentrations in the field distribution in a comparison using toxicity data and field observations of macroinvertebrates and fish from eastern Australia and suggests that LC_{50} values are useful indicators of upper salinity tolerance values in the field.

3.1.7 Relevant endpoints for determining the chronic toxicity of salts

The biological response chosen for a toxicity test should be one that is ecologically significant in order that the results of the test may be applied to management of an

aquatic ecosystem. The OECD (1998) stressed the ecological relevance of survival, growth and reproduction as toxicity test endpoints. According to Warne (2001), only toxicity data measuring ecologically important endpoints were used to compile the Australian and New Zealand Water Quality Guidelines for Toxicants (ANZECC and ARMCANZ 2000).

3.1.8 Overview of Caridina nilotica distribution, life history and biology

Caridina nilotica occur in the rivers and lakes of southern Africa (Hart, 1981). The genus *Caridina* is the only member of the Atyid shrimps in southern Africa (Hart, 1982). According to Barnard (1950) quoted by Hart (1983), the distribution of *C.nilotica* in South Africa extends as far south as the Umzimvubu river and as far west as Lake Ngami. Hart (1983) stated that specimens of *C.nilotica* had been collected in the Keiskamma and Bushmans River (Eastern Cape), representing a southerly extension of its distribution. In 1984, a further southerly extension to *C.nilotica* distribution was determined when specimens were found in the Gamtoos River (Coetzee, 1985). Hart (1983) found that *C.nilotica* have a temperature activity range of 11,5 to 31,5 ˚ C and concluded that temperature tolerance may be a significant factor affecting this organisms' distribution. In southern Africa, shrimp of the genus *Caridina* are ecologically important as herbivores and scavengers (Hart, 1981). *C.nilotica* has a specialised feeding system and feeds as a scraper of surfaces of organic macrophytes and other substrates, feeding on microbiota (Hart, 1980). As scavengers, the species may enhance the production of submerged macrophytes by removing debris and epiphytic microphlora (Hart, 1981). These shrimp may also be an important source of food for small fish (Hughes, 1992). *C.nilotica* comprises a significant proportion of zooplankton in Lake Victoria (Hart, 2001, quoting Lehman *et al.*, 1996). Time from 'egg to egg' takes approximately three months (personal observation and Muller, pers.comm., 2004) and individuals transform from male to female with increasing age (Okuthe *et al*., 2004).

3.1.9 Are salts toxicants?

Although salts are natural physiological stressors, exposure of salts at a high concentration to test organisms have shown classic toxicant concentration – response curves. This is shown in a study by Kefford *et al*. (2002). This is also evident in toxicological tests run by the UCEWQ using NaCl and Na_2SO_4. Much of the

concentration response data for salts fit classic toxicological models such as Probit. The Australian and New Zealand Environmental Conservation Council (ANZECC) also classify salinity as a stressor that is directly toxic to biota (Kefford *et al*., 2002).

It is also possible that certain freshwater faunal species flourish best at a salinity level that is above that of deionised water. Therefore dissolved salt at a low concentration may be beneficial to certain freshwater species while dissolved salts may become toxic at higher concentrations. This could complicate matters in salinity toxicity testing using certain species as negative effects in the control may be higher than effects at the lower salinity levels. It was found that a certain lifecycle stage of larvae of the Cape River Shrimp (*Palaemon capensis*) require salinities in the range of 10 – 25 parts per thousand to develop (Coetzee, 1989). *C.nilotica* individuals have also been found in fairly brackish waters of 4 parts per thousand and 6 parts per thousand in the Keiskamma and Bushman's rivers respectively (Hart, 1983).

3.1.10 Aims of this chapter

The aim of this chapter is to develop a method for undertaking chronic toxicity tests on an early life-history stage of *C.nilotica*, in order to undertake toxicity tests using selected toxicants. The toxicants tested were NaCl and Na_2SO_4, indicative of natural and agriculturally caused salinisation and mining and industry caused salinisation respectively (Scherman *et al*., 2003). The statistical relationship between NOECs and LC_x values for mortality determined in the chronic tests was explored. The possible use of *C.nilotica* juveniles in a short-term chronic test was discussed.

3.2 Methods

Initial experiments in 2003 were used to develop and refine a successful protocol for chronic testing using *C.nilotica* and as range finding tests for determining a tolerance range for NaCl and Na_2SO_4. The outcomes of these experiments included the realisation that young *C.nilotica* juveniles appear to have a high natural attrition rate, leading to unacceptable control mortalities if used in chronic tests. These initial experiments also helped to determine a suitable concentration range for NaCl and the development of an appropriate feeding protocol. A method for measuring growth as a

biological response of this species was refined. The methods and results of these initial tests are not recorded here as the tests were reproduced using the more refined protocol in 2004 and subsequently yielded better results.

3.2.1 Life stages used

The chronic toxicity test developed using *C.nilotica* is essentially a partial life-cycle test. At test initialisation, juveniles of approximately 45 days in age are used. The exposure period ended when females became gravid and was approximately 80 days as most individuals started developing eggs within 90 days of age (Muller *pers.comm.*, 2003). At this stage individuals are approximately 125 days of age. No chronic tests using *C.nilotica* embryos were undertaken. No acclimation periods were implemented as test organisms were obtained from a laboratory culture which was maintained under similar temperature and light regimes.

As there is no way of determining the sex of young juveniles except in a destructive manner (Hart, 1980), there was no determination of the sex ratio of the starting juvelines in the chronic tests. Juvelines were transferred to the vessels in a random manner and therefore it was assumed that the male:female ratio in the tanks was 50:50.

The number of animals used in each vessel was dependant on the number of available animals at the time of the start of each chronic toxicity test. The actual numbers used are indicated in the results section of this chapter in Table 3.4.

3.2.2 Biological response measured

Ecologically important biological responses such as survival, growth and reproduction should be measured in a chronic toxicity test (Warne, 1998; Giedy and Graney, 1989). It was decided to focus on survival, growth and reproduction as measurable biological responses in this study.

Survival was measured by counting and recording dead individuals in the treatments daily and removing them from the experimental chambers.

The effects of the salts on growth rate of *C.nilotica* may be as a result of energy within individuals being spent on detoxification of the toxicant rather than growth (Giedy and Graney, 1989; Lanno *et a1*.,1989). Carapace length measurements as an indication of growth were made by measuring the distance laterally from the anterior margin of the carapace behind the insertion of the eyestalk to the most posterior margin of the carapace (Hart, 1980). At set intervals, 20 individuals (or as many as were available if less than 20 remained) were removed from the experimental vessels and their carapace lengths measured. All individuals from a particular treatment were placed in a petri-dish with some of the original treatment solution. The petri-dish was placed into a shallow container containing ice-cold water and finely crushed ice. The container was placed under a microscope fitted with a micrometer. As individuals ceased movement due to the cold temperature, carapace lengths were readily measured and the individuals returned to the relevant treatment vessel within the experiment. This method of measurement is non - destructive and non - intrusive, as indicated by low control mortalities within the chronic tests. Carapace length measurements occurred at set intervals, namely on day 0,4,10,24,38,52,66 and 80. These intervals were chosen arbitrarily although shorter intervals were chosen in the earlier stages of the test as growth rates are higher in young juveniles (Hart, 1980).

In addition to carapace length measures, growth was estimated by measuring dry weights of individuals. Dry weights were taken at the end of experiments, as this measure, in contrast to carapace measures, is destructive. Dry weights of individuals were undertaken by oven drying the organisms for 48 hours at 105 ˚C. Brower *et al.* (1990) suggested drying at 105 ˚C until the weight of the samples stabilise. However, since the chronic test concerns relative dry weights of individuals and not absolute biomass measures, a constant time of drying of 48 hours was chosen. Females were removed from the experiment at the first sign of being gravid, and their carapace length and dry weight measured. It is unlikely that females will experience significant growth after becoming gravid as the formation and maintenance of the eggs will consume the majority of growth energy available (Hart, 1980).

Reproductive endpoints were also measured. Females were removed from the experiment at first sign of carrying eggs. The eggs were removed from the female using a soft paintbrush and overall number of eggs per female counted.

3.2.3 Concentration range

Three replicated concentration ranges of 8 treatments and 1 control were used in the experiments for both NaCl and Na_2SO_4. All three replicates of each chronic test were not started at the same time, but staggered in time as sufficient young *C.nilotica* juveniles became available. Generally subsequent replicates within a test started within two weeks of each other.

In the initial method development in 2003, it was found that the selected concentration range for NaCl was not wide enough. In 2004, the concentration range was estimated from the results of Two-Step Linear Regression (Mayer *et al*., 1994) acute to chronic data extrapolation and from the available acute and short – term chronic data contained on the IWR/UCEWQ toxicity database. The first replicate of this test had the following concentration range: 0 mg/L, 1300 mg/L, 1900 mg/L, 2700 mg/L, 3800 mg/L, 5500 mg/L, 7800 mg/L, 11200 mg/L and 16000 mg/L. The range was found to be too high and subsequent replicates had the following concentration range: 0 mg/L, 1300 mg/L, 1900 mg/L, 2200 mg/L, 2500 mg/L, 2700 mg/L, 3300 mg/L, 3800 mg/L and 5500 mg/L.

The concentration range for Na_2SO_4 was also determined by exploring the short-term chronic toxicity data on the IWR/UCEWQ database and from the results of Two-Step Linear Regression (Mayer *et al*., 1884) acute to chronic data extrapolation. The treatments chosen were 0 mg/l, 800 mg/L, 1070 mg/L, 1400 mg/L, 1900 mg/L, 2530 mg/L, 3375 mg/L, 4500 mg/L and 6000 mg/L. Test solutions for both toxicants were made up by dissolving analytical grade salts in dechlorinated tap water.

3.2.4 Experimental vessels and experimental set-up

Experimental vessels used were rectangular glass tanks of approximately 14 litres in volume (Figures 3.1 and 3.2). 12 litres of diluent was placed in each tank. The shrimp were kept free swimming in these vessels. Netting was suspended within each tank as substrate for the neonates and to prevent individuals from being washed around the vessel. A ceramic tile was also placed in each vessel as an attachment surface for the neonates. Cling wrap was placed over the tank top openings to prevent excessive evaporation and to prevent individuals from jumping out of the tanks.

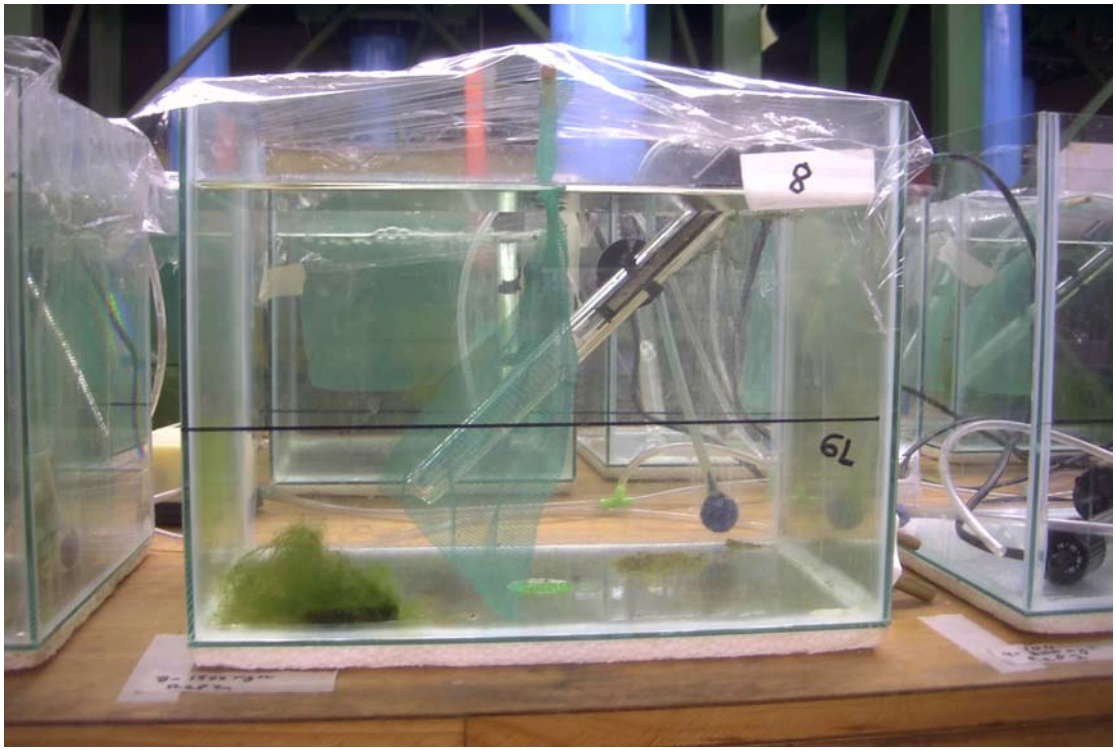


Figure 3.1 Experimental vessel used in the *C.nilotica* chronic toxicity test.

Figure 3.2 Photograph of the entire laboratory showing all three replicated concentration ranges.

3.2.5 Environmental control

Temperature of the laboratory was controlled by air conditioning maintained at approximately 22 (+-2°C) °C. Temperature of the vessels was maintained at approximately 24 °C (+-1°C) using aquarium heaters. A light – dark regime of 12/12 hours was used. No filters were used within the vessels but concentrations were renewed once a week, following a static-renewal protocol as defined by Rand *et al*., (1995). All vessels were aerated using air stones attached to aquarium air pumps with rubber tubing.

3.2.6 Feeding protocol

Cooney (1995) recommends that a specific feeding protocol outlining food type, ration and frequency of feeding be compiled for chronic tests. The selected feeding regime can affect the metabolism of the test animals, which may in turn affect the rate of uptake, absorption and depuration of the toxicant (Lanno *et al*., 1989). The selected feeding regime may also affect the biochemical composition of the test organism, which could affect their response to a toxicant (Lanno *et al*., 1989).

C.nilotica individuals were fed algal flakes ground to a fine powder. Faecal pellets were left in the tanks as algae or bacteria growing on the pellets are a source of food that the shrimp continually scrape (Hart, 1980). Additionally, a ceramic tile seeded with algae was placed in each experimental vessel.

It was decided to use the '*feeding ad libitum*' feeding protocol method (Lanno *et al*., 1989). This is basically 'feeding to satiety' i.e. enough food for the test organisms to consume until satisfaction. A method to ensure that '*feeding ad libitum*' is occurring as suggested by Muller (*pers.comm.*, 2004) is to examine the transparent feeding canal of the animals on a daily basis. If the test organisms are indeed feeding to satiety then food will be visible in the alimentary canal. To determine whether the individuals were being provided with enough food, the alimentary canals of the shrimp were observed daily during mortality readings. If it appeared that many of the individuals had empty alimentary canals, the amount of food going into the experimental vessel was increased. Animals in the test were fed every day after

mortality observations. A small amount of ground algal flakes was placed into each vessel using forceps, ensuring that the food sank to the bottom.

3.2.7 Environmental test system measures

Temperature of the water within the vessels was measured every day using thermometers during the daily mortality readings. The minimum and maximum air temperatures of the laboratory were also recorded daily. Treatment concentrations were changed on a weekly basis. Before and after each water change, Electrical Conductivity and Total Dissolved Solids (Cyberscan 200 conductivity meter), Dissolved Oxygen (Wissenschaftlich Technische Werkstätten OXI 92 dissolved oxygen meter), pH (Cyberscan pH 10 meter) and light intensity were measured. Water samples were taken periodically for measurement of pH, alkalinity, ammonium, nitrate and nitrite, fluoride, sodium, magnesium, silicon, phosphate, sulphate, chloride, potassium, calcium, Electrical Conductivity and Total Dissolved Solids, barium, boron, vanadium, lead, cadmium, molybdenum, strontium, zinc, copper, nickel, iron, manganese, chromium and aluminium. Water sample analysis was done by Resource Quality Services (RQS), Kwamhlanga Road, Roodeplaat Dam in South Africa.

3.2.8 Statistical analysis

Grouped mortality results for all three replicates within each experiment were analysed using ANOVA or a non-parametric alternative in Statistica Version 6 (StatSoft, 2002). Additionally, mortality results for each replicate were analysed separately using appropriate regression models: the probit software Probit (USEPA, 1993) and least square linear regression (in Statistica Version 6 (StatSoft, 2002)). Linear regression was also applied to the mortality data of all three replicates grouped. An LC_5 from the regression line was compared to the estimated NOEC.

Carapace length measurements, dry weights and number of eggs per individual were analysed separately for each replicate using ANOVA or a non-parametric alternative in Statistica Version 6 (StatSoft, 2002). In addition, the grouped results from all three replicates for percentage of pregnant survivors and number of eggs per individual were analyzed using ANOVA or a non-parametric alternative in Statistica Version 6 (StatSoft, 2002). Grouped results for all three replicates were used in the case of the

percentage of pregnant survivors data and percent mortality data as only one data point per treatment within a replicate is possible with a percentage calculation, and multiple points per treatment are needed for an ANOVA statistical test (Crane *et al*., 2000).

An ANOVA and parametric post-hoc statistical methods assume that the data have equal variance and that the data follow a normal distribution (Crane *et al*., 2000). The following data transformations (ZAR, 1974) were used if the untransformed data did not fulfill the requirements of parametric tests:

1. The logarithmic transformation where $X' = \log_{10}(X + 1)$.
2. The arcsine transformation where $X' = \arcsin X^{1/2}$.
3. The square root transformation where $X' = (X + 0.5)^{1/2}$.

Where X is the untransformed data and X' is the transformed data.

Non-parametric alternatives were used if the transformations did not yield data that fulfill requirements for parametric statistical analysis.

The collected mortality results of the three replicates were applied to regression models if the LC_{50} values produced in the three replicates were not statistically significantly different from each other. A method by APHA (1992) cited in Muller and Palmer (2002) to determine if LC_{50} values are significantly different from each other was used:

$$f_{1.2.} = anti \log ((\log f_1)^2 + \log(f_2)^2)^{½}$$

where f = factor for 95% confidence limits, i.e. upper 95%/LC_{50}

if the ratio of the greater LC_{50}:lower LC_{50} is greater than $f_{1.2.}$ then the LC_{50}s are significantly different.

The NOEC values determined in the chronic experiment were compared to the LC_5 value determined from the regression model, since Warne (1998) associates the LC_5

determined from regression as being equivalent to an NOEC determined by hypothesis testing via ANOVA statistical testing. An LC_x value that corresponds to the NOEC value for each salt was also determined if the LC_5 was not equivalent to the NOEC.

3.3 Results

3.3.1 Chronic NaCl experiment

3.3.1.1 Mortality

Generally the chronic survival response of C.nilotica to NaCl over the full 80 days exposure period were similar for all three replicates (Table 3.1.). All treatments under 2700 mg/L showed low mortality. It appears that the 2700 mg/L treatment may be a threshold where increased mortality begins to appear, with the relatively similar 2500 mg/L treatment showing low mortality. The means of mortality response categorised by treatment over the exposure period for all replicates grouped are plotted with standard deviations indicated as whisker – plots around the means (Figure 3.3). It is obvious that the 2700 mg/L treatment showed significantly higher mortality than the control over the entire exposure period. Grouped mortality data for all three replicates at 80 days were analysed for statistical differences between treatments (sample sizes depicted in Table 3.4). The log transformed mortality data showed the highest normality in distribution (Shapiro-Wilk W = 0,85, $p < 0,05$). None of the transformations used produced homogeneous data as indicated by the Levene's Test ($p < 0,05$). A non – parametric Kruskal – Wallis Test showed no significant differences among the treatments ($p > 0,05$). It was evident however from Figure 3.3 that the 2700 mg/L treatment was significantly different from the control. The LOEC for mortality was therefore the 2700 mg/L treatment and the NOEC was the 1900 mg/L treatment.

Mortality results for each replicate at 80 days exposure period were analysed using the Probit (USEPA, 1993) model. The Probit model was not suitable for the first two replicates as indicated by a chi^2 value. The mortality data was therefore analysed using least square linear regression (Figure 3.4). The LC_{50} for the first replicate was estimated to be 2425 mg/L. Linear regression did not fit the mortality results of the

first replicate particularly well with an r^2 of 0,57. Since a positive relationship between NaCl concentration and mortality was only evident from the 1900 mg/L treatment, a straight line was fitted to the results of the 1300 to 3300 mg/L treatment, thereby ignoring the mortality results in the control (Figure 3.4 B).The LC_{50} for the second replicate was estimated to be 2490 mg/L. The mortality results of the third replicate fitted the Probit model (Figure 3.5) as indicated by a chi^2 value. The LC_{50} as estimated by the Probit model was 2637 mg/L. Control mortalities were acceptable for all replicated concentration ranges except that of replicate three (Table 3.1), if the boundary of 20 % control mortality for chronic toxicity tests (Cooney, 1995) is accepted. Control mortality in replicate 3 started after day 40 (Figure 3.3). The reason for mortality in the control of replicate 3 could not be established.

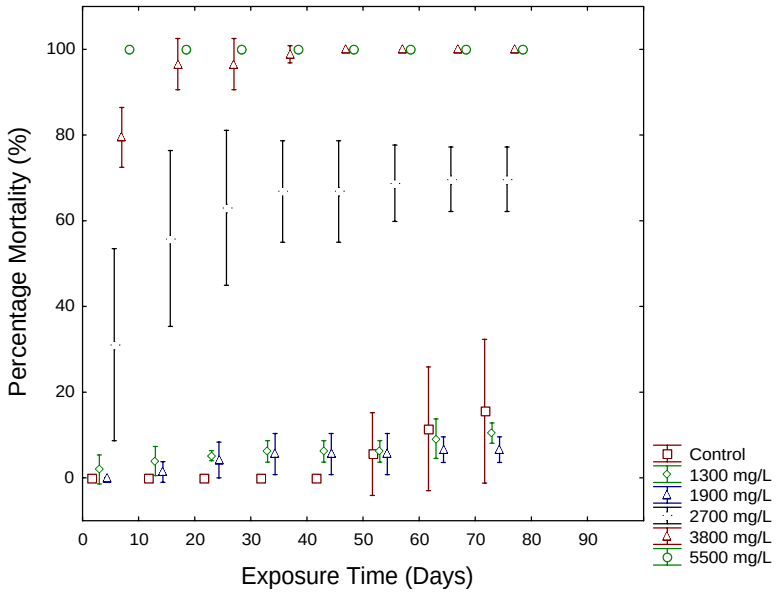


Figure 3.3 Means and standard deviations of the grouped mortality results of all replicated concentration ranges in the NaCl chronic toxicity test.

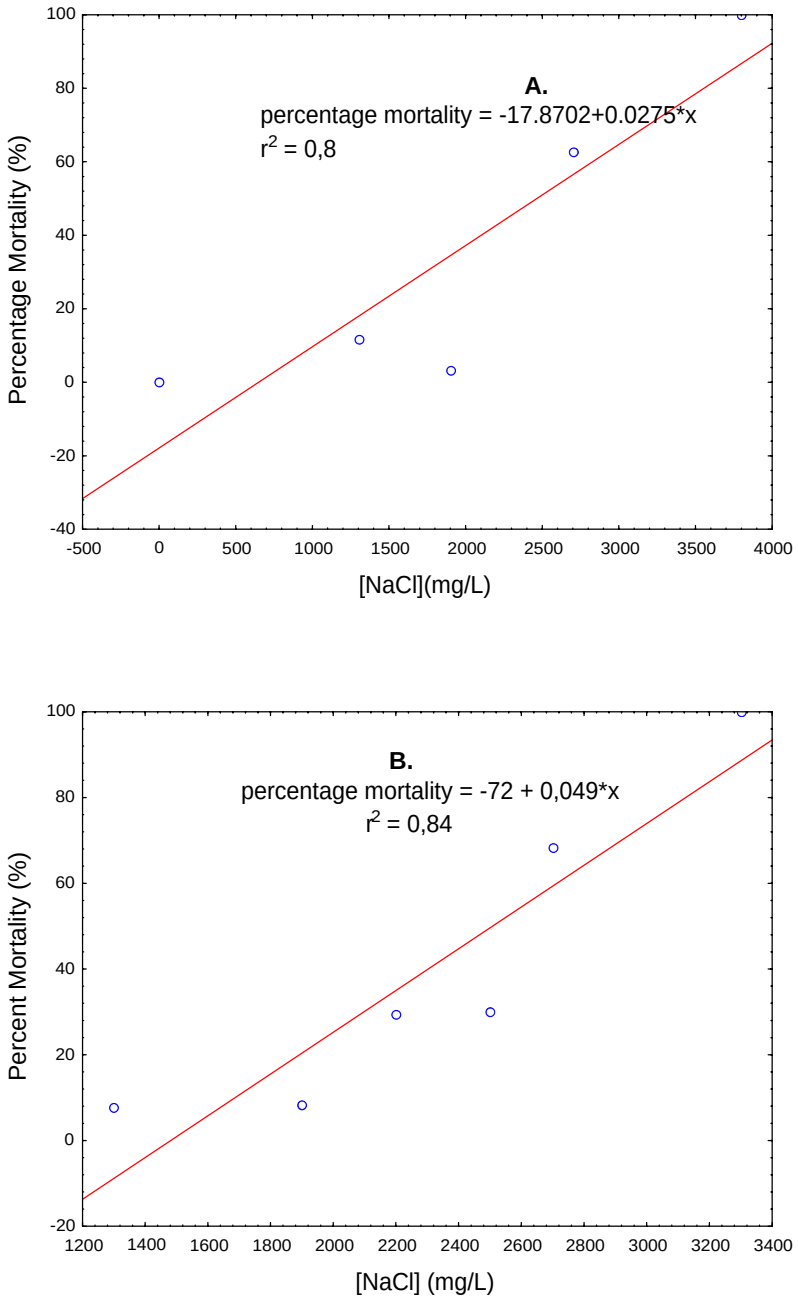


Figure 3.4 Regression lines fitted to accumulated mortality response over time for two replicated concentration ranges in the NaCl chronic experiment. Regression equations as well as goodness of fit (r^2) are indicated (Figure A - replicate 1 and Figure B - replicate 2).

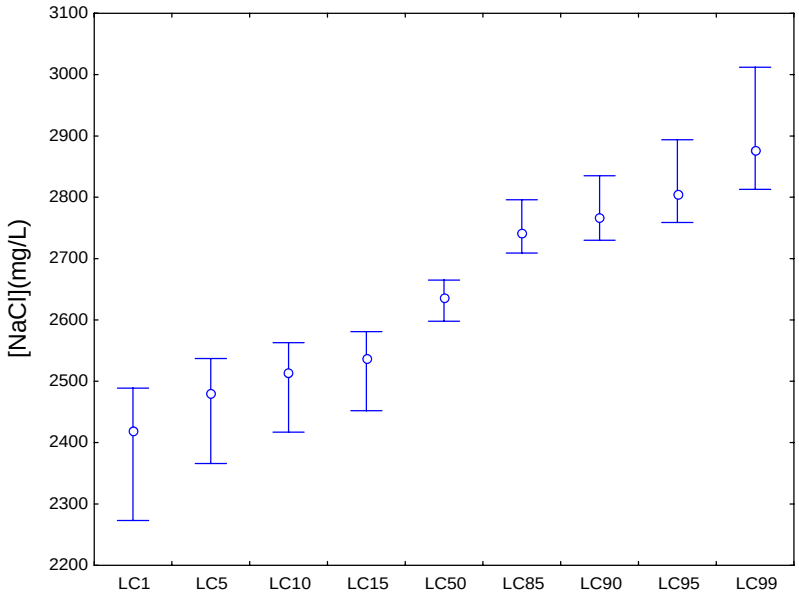


Figure 3.5 The LC_x and 95% confidence intervals of the third replicated concentration range mortality results of the NaCl chronic toxicity test analysed using the Probit model (Probit Program Version 1.5).

Table 3.1 Control mortalities and LC_{50} values determined using regression models in the NaCl chronic toxicity test. '*' - Could not be calculated.

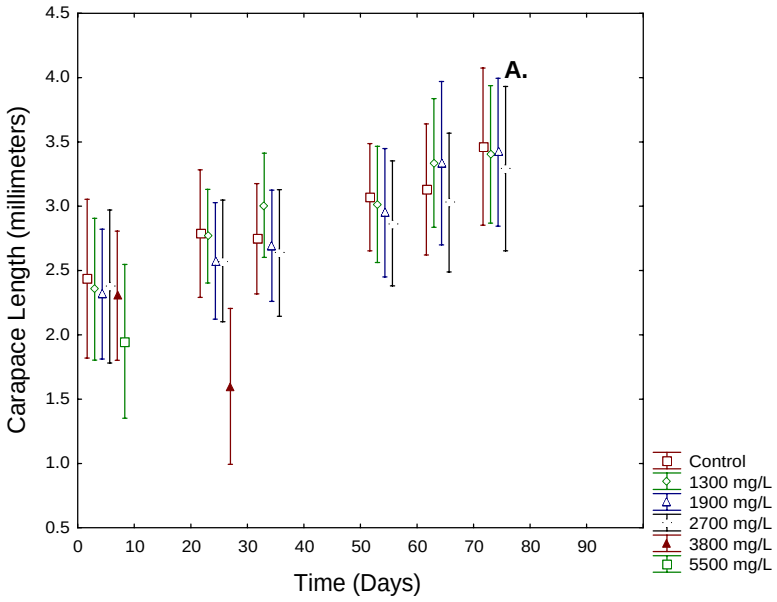
Replicate	LC_{50} mg/L	95 % Confidence limits		%Control mortality
		Upper	Lower	
1	2425	*	1700	0
2	2490	3200	2120	13.3
3	2637	2665	2598	33.3

3.3.1.2 Growth

Growth results are shown in Figure 3.6 with means and standard deviations of carapace length analysed separately for each replicate. Carapace lengths for all three replicates were not grouped for analyses, as there were inter-replicate differences in the size of the young juveniles at the start of each replicate (Figure 3.6 A - C), which may have affected the results of statistical analysis. Differences between treatments

were tested for at exposure durations where the standard deviations of the treatments did not appear to overlap (Figure 3.6 A – C), although differences between treatments at the full 80 days duration were tested for if non-overlapping of standard deviations persisted for the full exposure duration. No carapace growth measurements were possible for treatments where there was 100% mortality.

There did not seem to be any significant differences between treatments in the first replicate (Fig 3.6 A). Replicate 2 showed a significant decrease in mean carapace length with increasing salt concentration. However, carapace length data between treatments appears to show significant difference only on day 80 (Figure 3.6 B) and therefore day 80 data were analysed for significant differences between treatments (sample sizes depicted in Table 3.4). The untransformed carapace length data showed the highest degree of normality in distribution (Shapiro-Wilk $W = 0,96$, $p < 0,05$) but none of the data transformations used produced homogeneous data as determined using the Levene's test ($p < 0,05$). A non – parametric Kruskal – Wallis test showed that the 2700 mg/L treatment was significantly different from the control on day 80 ($p < 0,05$). Therefore the LOEC for carapace length of replicate 2 was the 2700 mg/L treatment and the NOEC was the 2500 mg/L treatment. Replicate three also showed a trend of decreasing carapace length with increasing NaCl concentration, but the trend appears significant from much earlier in the test. Statistical differences between treatments on day 80 were investigated (sample sizes depicted in Table 3.4). The log transformation showed the highest degree of normality in distribution (Shapiro-Wilk $W = 0,96$, $p < 0,05$) but none of the transformations produced homogeneous data as indicated by the Levene's Test ($p < 0,05$). A non – parametric Kruskal – Wallis Test ($p < 0,05$) showed that all treatments from 2200 mg/L and higher were significantly different from the control on day 80. The LOEC for carapace length of replicate 3 was therefore the 2200 mg/L treatment and the NOEC was the 1900 mg/L treatment.



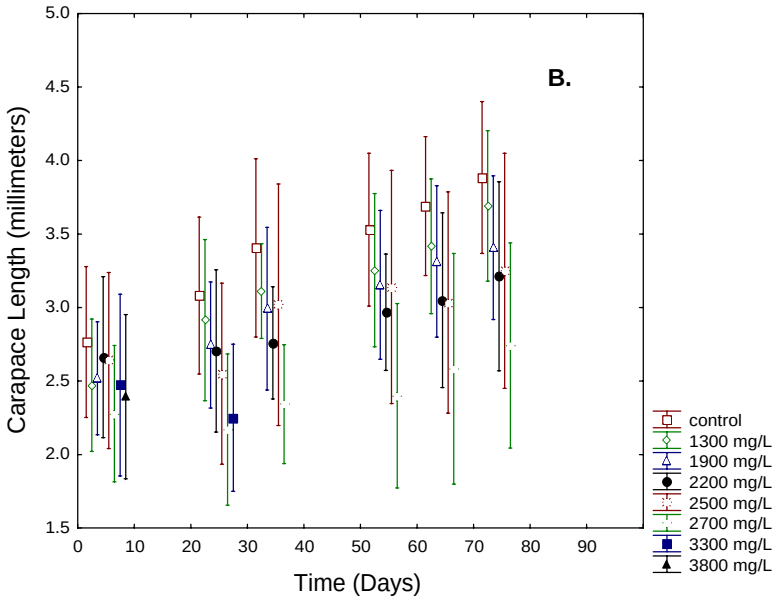


Figure 3.6 Means and standard deviations of carapace length results at each of the sampling periods for the three replicated concentration ranges of the NaCl chronic toxicity experiment (Figure A - replicate 1, Figure B - replicate 2 and Figure C - replicate 3).

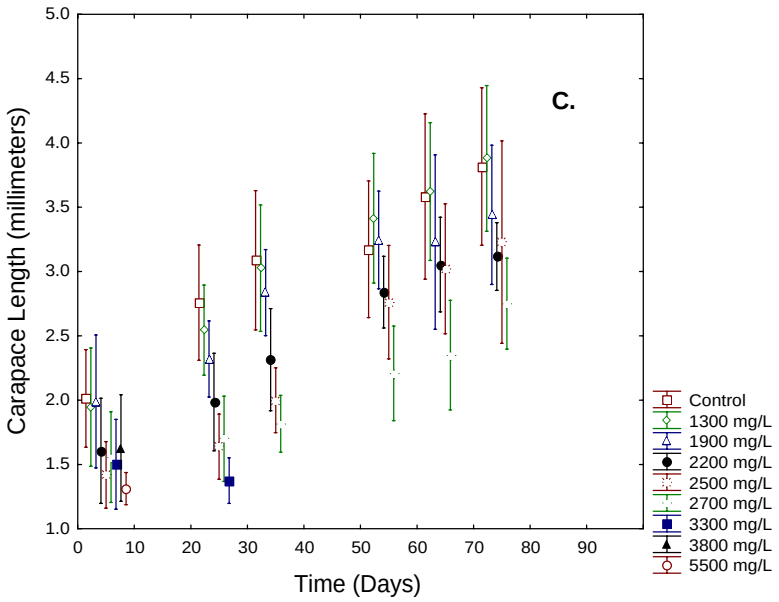
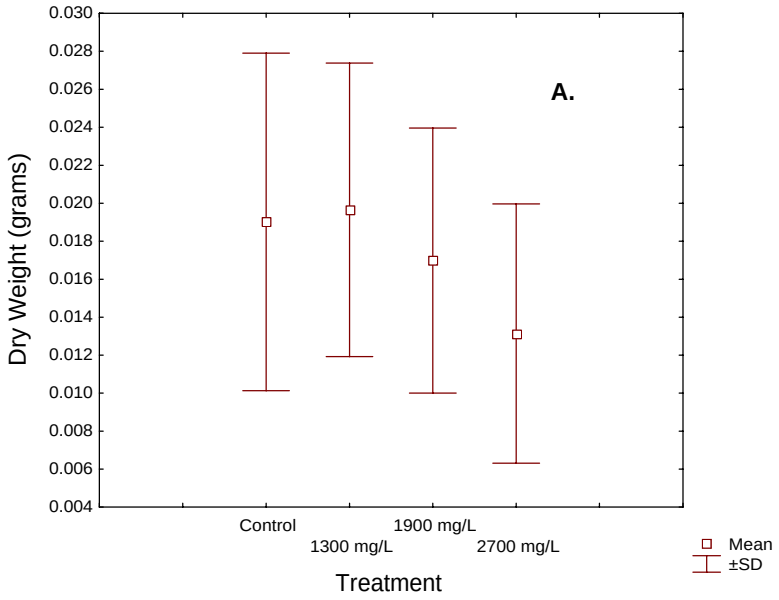


Figure 3.6 continued. Means and standard deviations of carapace length results at each of the sampling periods for the three replicated concentration ranges of the NaCl chronic toxicity experiment (Figure A - replicate 1, Figure B - replicate 2 and Figure C - replicate 3).

The means and standard deviations of dry weight results for the three replicates are represented in Figure 3.7. There did not appear to be any obviously significant differences between treatments for replicate 1 (Figure 3.7 A) and 3 (Figure 3.7 C). Figure 3.7 B did however indicate significant differences among treatments in replicate 2 (sample sizes depicted in Table 3.4). A Levene's test ($p < 0,05$) indicated that the untransformed data had equal variance. The untransformed data has the closest distribution to normality (Shapiro-Wilk $W = 0,98$, $p < 0,05$). An ANOVA indicated the presence of a significant difference between treatments ($p < 0,05$) and a parametric Tukey test indicated that the 2200 mg/L treatment and all higher treatments were significantly different ($p < 0,05$) from the control. Therefore the 2200 mg/L treatment was taken as the LOEC and the 1900 mg/L treatment as the NOEC for the dry weight results in replicate 2.



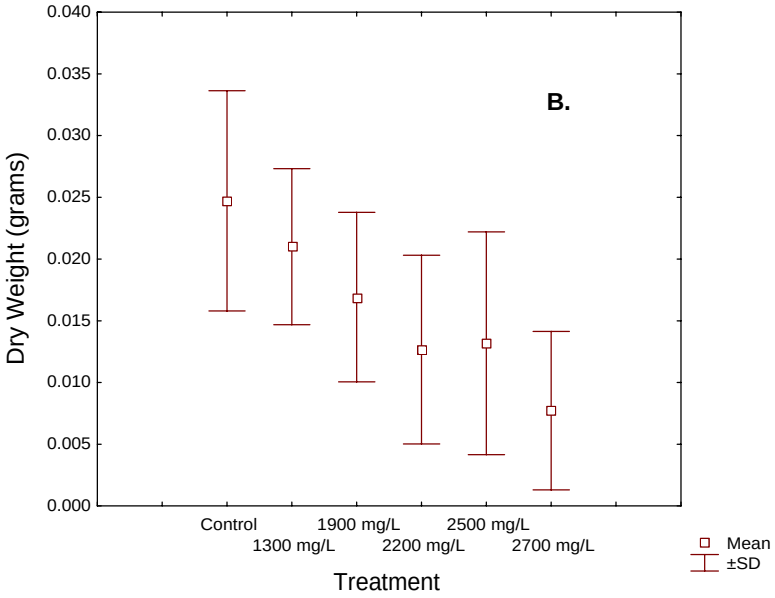


Figure 3.7 Means and standard deviations of growth (dry weight in grams) results for the three replicated concentration ranges from the NaCl chronic toxicity experiment (Figure A - replicate 1, Figure B - replicate 2 and Figure C - replicate 3).

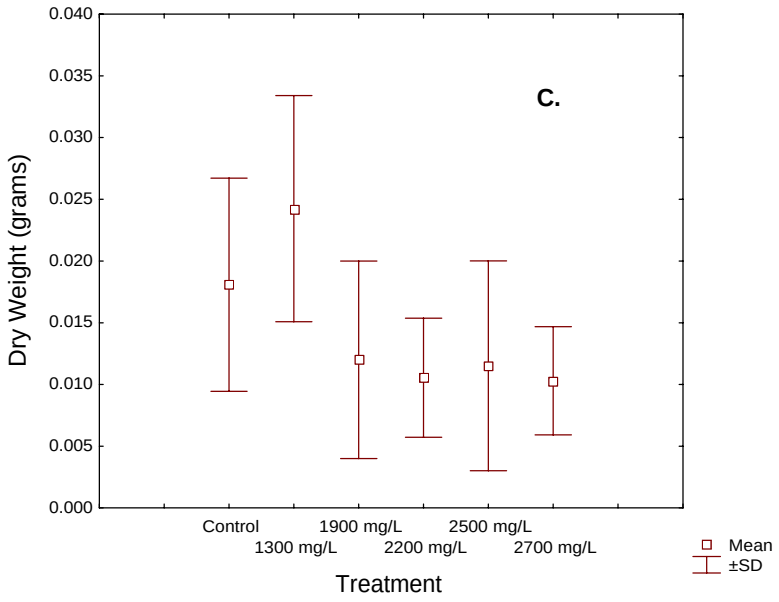


Figure 3.7 continued Means and standard deviations of growth (dry weight in grams) results for the three replicated concentration ranges from the NaCl chronic toxicity experiment (Figure A - replicate 1, Figure B - replicate 2 and Figure C - replicate 3).

3.3.1.3 Reproduction

The number of gravid females appearing in a treatment was taken as a percentage of total survivors per treatment. The results from all three replicated concentration ranges collected showing the mean and standard deviations of the percentage of the survivors that were gravid is depicted in Figure 3.8. Figure 3.8 indicated that the 2700 mg/L treatment had significantly fewer gravid females compared to the control (sample sizes depicted in Table 3.4). The log -transformed data had a distribution closest to normality (Shapiro-Wilk $W = 0,96$, $p < 0,05$). A Levene's test ($p < 0,05$) indicated that the log - transformed and untransformed data among treatments had equal variance. An ANOVA indicated that there was a significant difference among treatments ($p < 0,05$). A parametric Tukey test ($p < 0,05$) found that the 2700 mg/L treatment was significantly different from the control using the untransformed data. The LOEC for these data was the 2700 mg/L treatment while the NOEC was 1900 mg/L.

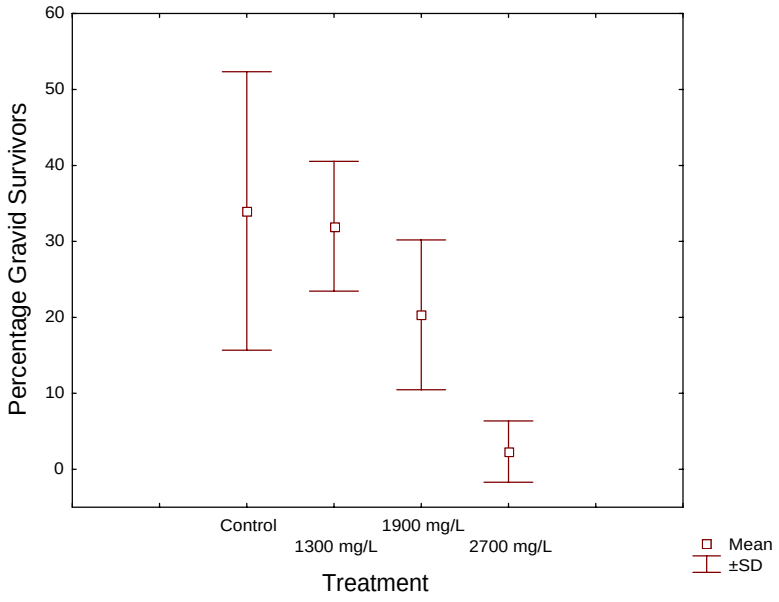


Figure 3.8 Means and standard deviations of reproduction (percentage gravid survivors) of the grouped results from all three replicated concentration ranges from the NaCl chronic toxicity experiment.

The mean number of eggs per female is shown in Figure 3.9, where the means and standard deviations are plotted separately for each replicate. No obvious differences were apparent although a decreasing egg number with increasing concentration was apparent. Differences between treatments for the three grouped replicates were also tested for. Figure 3.10 shows the means and standard deviations of number of eggs per female for the three replicates grouped. Here there were also no apparent significant differences. No eggs were found in the highest concentrations, either because of 100 % mortality or because no gravid females were evident in the highest concentrations.

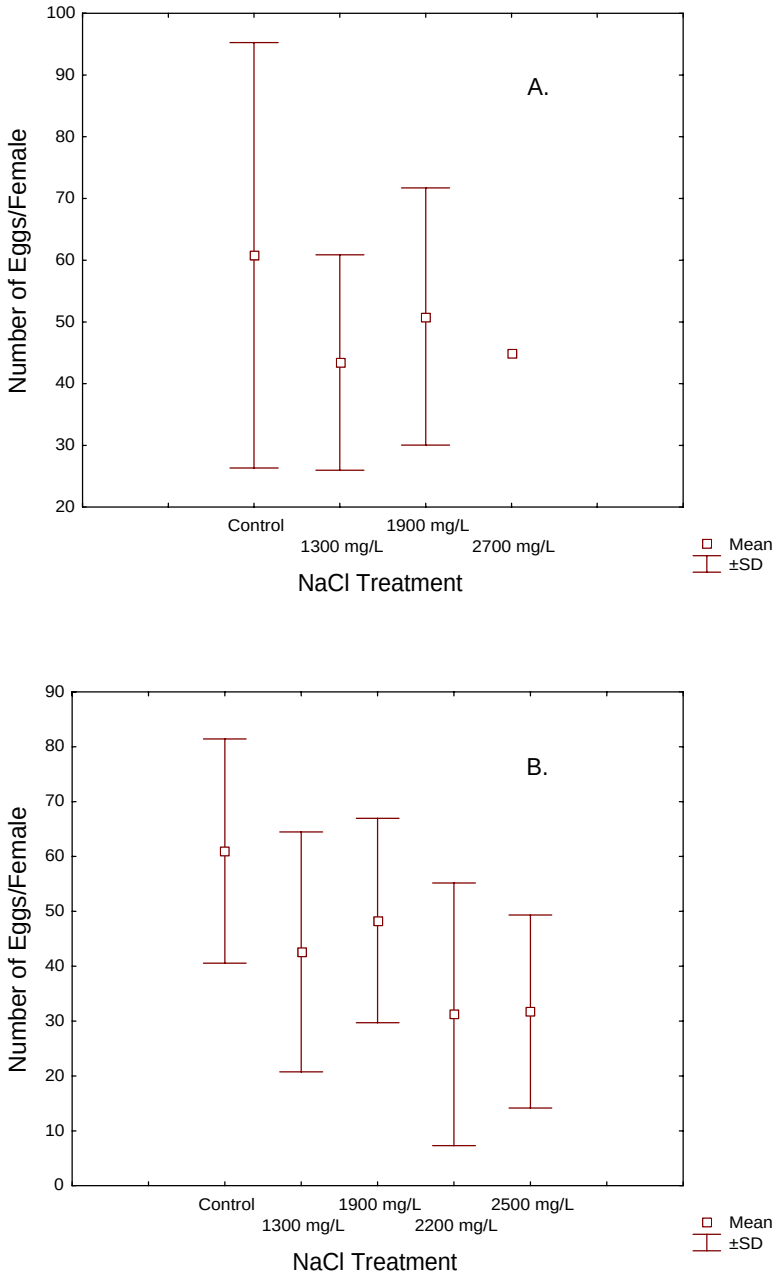


Figure 3.9 Means and standard deviations of reproduction (number of eggs per individual) results for the three replicated concentration ranges from the NaCl chronic toxicity experiment (Figure A - represents replicate 1, Figure B - replicate 2 and Figure C - replicate 3).

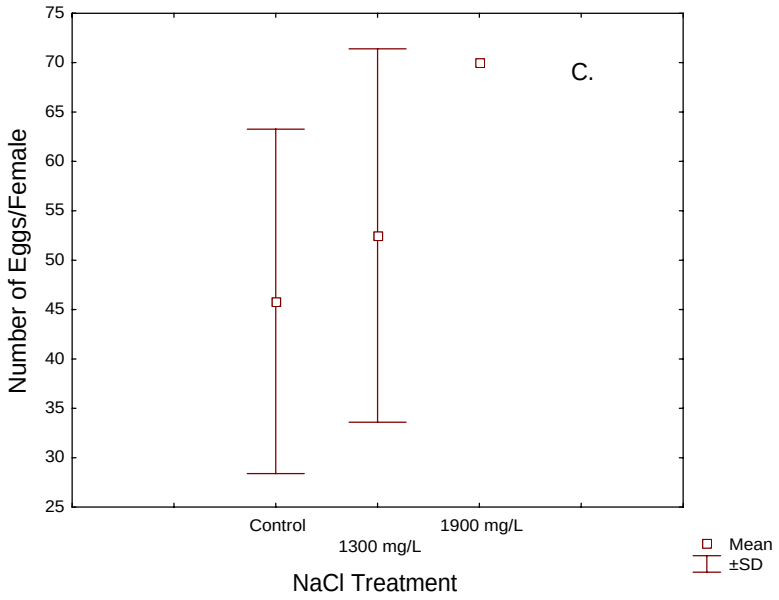


Figure 3.9 continued Means and standard deviations of reproduction (number of eggs per individual) results for the three replicated concentration ranges from the NaCl chronic toxicity experiment (Figure A - represents replicate 1, Figure B - replicate 2 and Figure C - replicate 3).

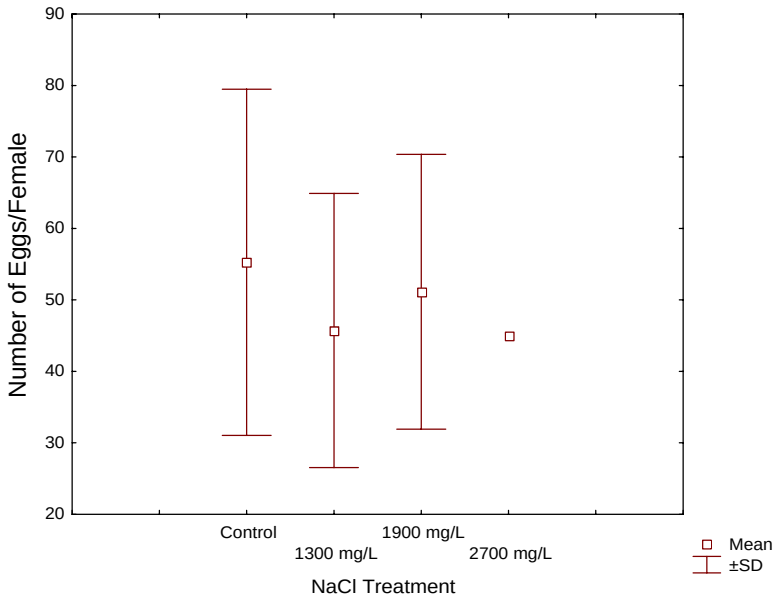


Figure 3.10 Means and standard deviations of reproduction (number of eggs) results for the collected results of all three replicated concentration ranges from the NaCl chronic toxicity experiment.

3.3.2 Chronic Na_2SO_4 experiment

3.3.2.1 Mortality

Unfortunately a heater burst during a weekly renewal of test mediums on day 30. This caused all remaining individuals in the 2530 mg/L treatment in replicate 3 to perish. The percentage mortality on day 30 in this treatment has been treated as the final mortality reading with the assumption that mortality would have stabilised to a large degree by this time. This is supported by the results of the preliminary method development chronic toxicity testing in 2003 where mortality generally stabilised after 15 days duration and the NaCl chronic toxicity test (Figure 3.3.) where mortality stabilised by 20 – 30 days.

Mortality results from all replicated concentration ranges were similar. Figure 3.11 depicts the collected mean and standard deviations of mortality results from all three replicated concentration ranges in the Na_2SO_4 chronic experiment. The 2530 mg/L treatment appears to be significantly different from the control in terms of the mortality response. Mortality data on day 80 was tested for significant differences between treatments (sample sizes depicted in Table 3.4). The untransformed data as well as all the data transformations could not produce homogeneous data as indicated by the Levene's Test ($p < 0,05$). A non- parametric Kruskal – Wallis test did not find any significant differences among treatments ($p > 0.05$) but it is evident (Figure 3.11) that the 2530 mg/L treatment is significantly different from the control. The mortality LOEC for these data was the 2530 mg/L treatment and the NOEC was the 1900 mg/L treatment.

None of the replicated concentration ranges produced data that fitted the Probit (USEPA, 1993) model. Linear regression of untransformed data produced the regression lines depicted in Figure 3.12. The first replicated concentration range produced an LC_{50} value of 2531.3, the second produced a LC_{50} value of 2802.9 and the third produced an LC_{50} value of 2669.5. LC_{50} values and control mortalities are depicted in Table 3.2.

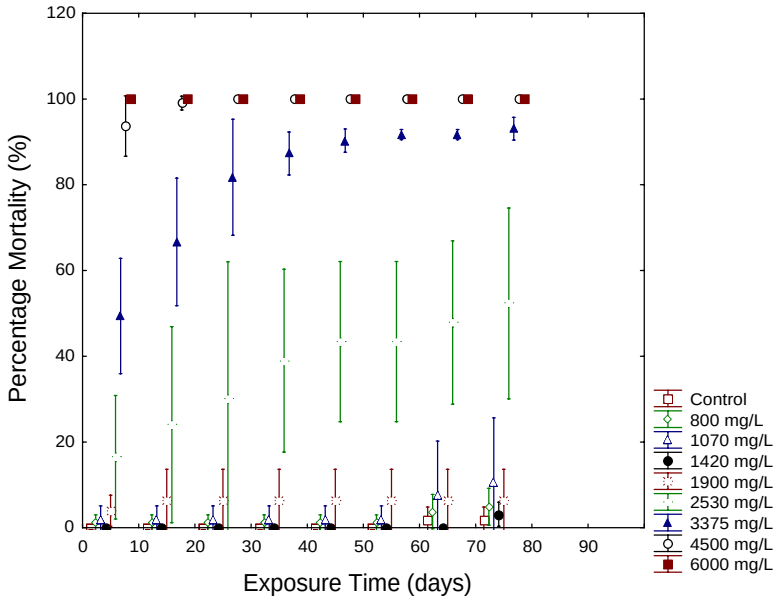


Figure 3.11 Means and standard deviations for the collected mortality results of the three replicated concentration ranges for the Na_2SO_4 chronic toxicity experiment.

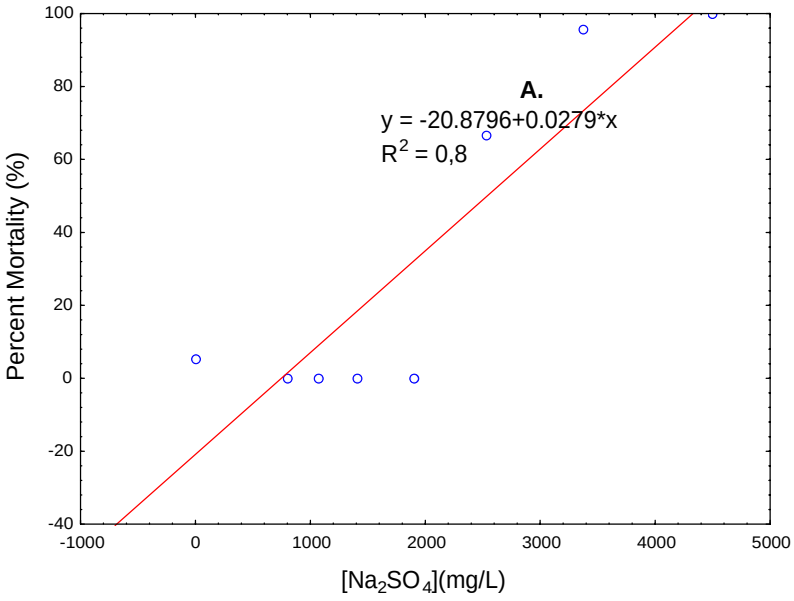
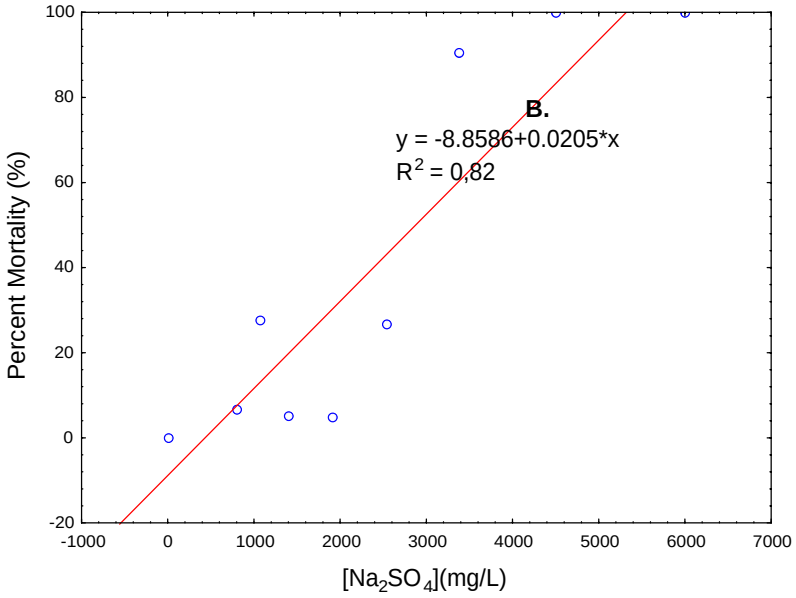


Figure 3.12 Regression lines fitted to accumulated mortality response over time for three replicated concentration ranges in the Na_2SO_4 chronic experiment. Regression equations as well as goodness of fit are indicated (Figure A - replicate 1, Figure B - replicate 2 and Figure C - replicate 3.



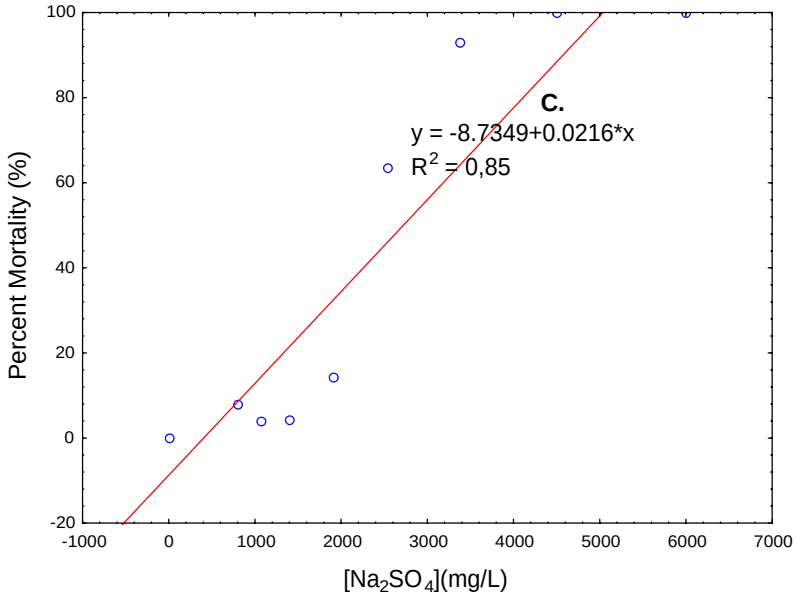


Figure 3.12 continued Regression lines fitted to accumulated mortality response over time for three replicated concentration ranges in the Na_2SO_4 chronic experiment. Regression equations as well as goodness of fit are indicated (Figure A - replicate 1, Figure B - replicate 2 and Figure C - replicate 3.

Table 3.2 Control mortalities and LC_{50} values determined using regression models in the Na_2SO_4 chronic toxicity test. '*' - Could not be calculated.

Replicate	LC_{50} mg/L	95 % Confidence limits		%Control mortality
		Upper	Lower	
1	2531	3800	1900	5.3
2	2803	4000	2200	0
3	2670	3500	2000	0

3.3.2.2 Growth

Since there were inter-replicate differences in the size of young juveniles at the start of each replicate (Figure 3.13 A – C), carapace lengths for all three replicates were not grouped together for analyses, but rather analysed separately for differences between treatments. Differences between treatments were tested for at exposure durations where the standard deviations of the treatments did not appear to overlap (Figure 3.13 A – C), although differences between treatments at the full 80 days duration were tested for if non-overlapping of standard deviations persisted for the full exposure duration. No carapace growth measurements were possible on treatments where there were 100% mortality.

Growth differences between treatments as shown by carapace length measurements were not as obvious as occurred in the NaCl experiment (Figure 3.13). It appears that the 3375 mg/L treatment may be significantly different from the control after day 30 in replicate 1 (Figure 3.13 A). Statistical differences between treatments according to carapace length measures on day 39 were explored (sample sizes depicted in Table 3.4). The log-transformed data had a distribution that was closest to normal (Shapiro-Wilk W = 0,82, $p < 0,05$). However the log transformation did not produce homogeneous data as indicated by a Levene's Test ($p < 0,05$). A non-parametric Kruskal – Wallis test indicated that the 3375 mg/L treatment was significantly different from the control ($p < 0,05$). The LOEC for carapace length data in replicate 1 was the 3375 mg/L treatment while the NOEC was the 2530 mg/L treatment.

None of the treatments in replicate 2 appear at any stage to be significantly different from the control (Figure 3.13 B).

It was highly unfortunate that the 2530 mg/L treatment in replicate 3 had to be abandoned after day 30 as reduced growth by day 20 – 30 (Figure 3.13 C) was evident in this treatment. Significant differences between treatments in terms of carapace length on day 23 were tested for, so as to include the 2530 mg/L treatment (sample sizes depicted in Table 3.4). Untransformed data showed a distribution closest to normality (Shapiro-Wilk $W = 0,97$, $p < 0,05$). A Levene's test showed that the untransformed data was homogeneous ($p < 0,05$). An ANOVA indicated a significant difference among treatments ($p < 0,05$), and a Tukey test indicated that the 1900 mg/L treatment and all higher treatments were significantly different from the control ($p < 0,05$). The LOEC for these data was the 1900 mg/L treatment and the NOEC was the 1400 mg/L treatment.

In some cases Figure 3.13 (A – C) does not show standard deviations of the treatments. This occurred in very high treatments where only one test animal was available for carapace measurement.

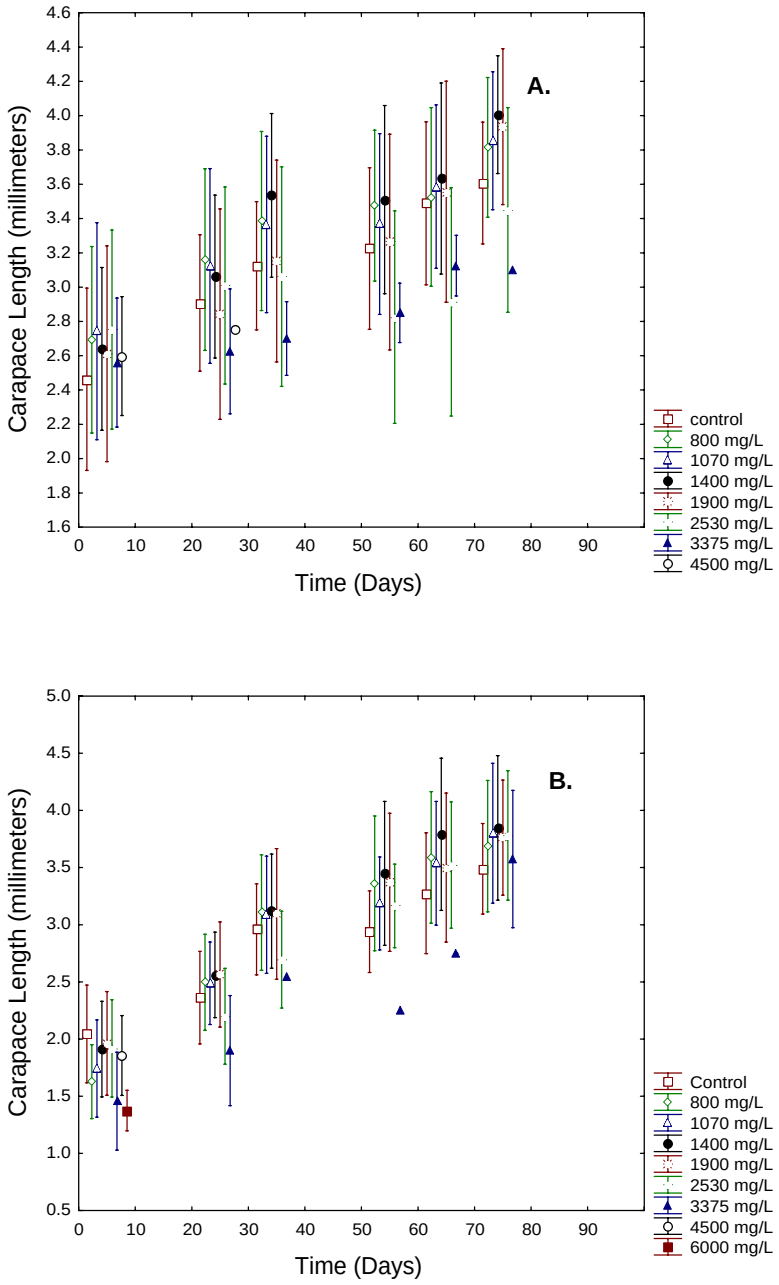


Figure 3.13 Means and standard deviations of growth (carapace length) results for the three replicated concentration ranges from the Na_2SO_4 chronic toxicity experiment (Figure A - replicate 1, Figure B - replicate 2 and Figure C - replicate 3).

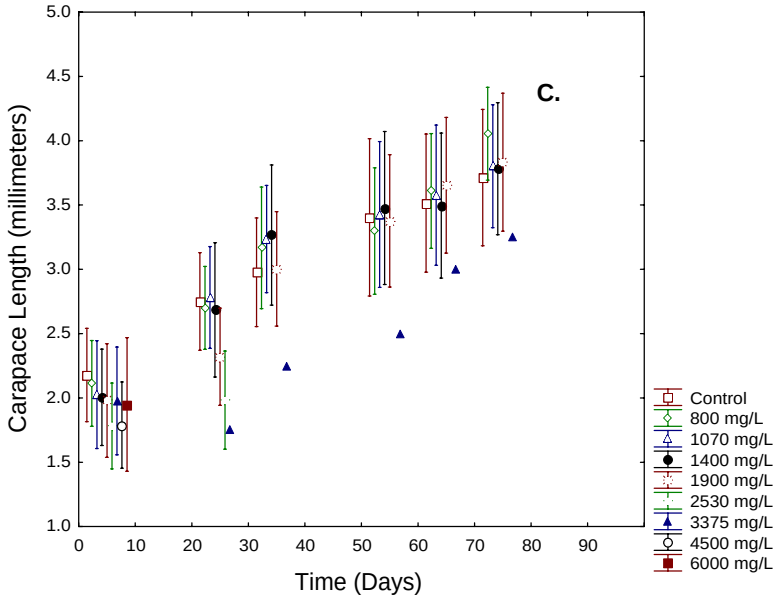


Figure 3.13 continued Means and standard deviations of growth (carapace length) results for the three replicated concentration ranges from the Na_2SO_4 chronic toxicity experiment (Figure A - replicate 1, Figure B - replicate 2 and Figure C - replicate 3).

Untransformed dry weight data of the first replicate (Figure 3.14. A) showed a normal distribution (Shapiro-Wilk W = 0,99, $p < 0,05$). A Levene's test indicated that the untransformed data was homogeneous ($p < 0,05$). An ANOVA indicated a significant difference among treatments ($p < 0,05$) but a Tukey test indicated that the 1400 mg/L treatment was positively statistically different from the control and therefore was not used as a Lowest Observed Effect Concentration (LOEC) (sample sizes depicted in Table 3.4). A non-parametric Kruskal – Wallis test however found that the 2530 mg/L and 3375 mg/L treatments were significantly different from the control ($p < 0,05$). The LOEC of these data was the 2530 mg/L treatment while the NOEC was the 1900 mg/L treatment.

Untransformed dry weight data from replicate 2 (Figure 3.14 B) appeared to follow a normal distribution (Shapiro-Wilk W = 0,93, $p < 0,05$) and a Levene's test indicated that the untransformed data was homogeneous. An ANOVA indicated a significant difference among treatments ($p < 0,05$) but a Tukey test found no treatments significantly different from the control ($p > 0,05$) (sample sizes depicted in Table 3.4). A non- parametric Kruskal – Wallis test indicated that the 1070 mg/L treatment was positively significantly different to the control while the 2530 mg/L and 3375 mg/L

treatments were negatively significantly different from the control ($p < 0,05$). The LOEC from these data was the 2530 mg/L treatment while the NOEC was the 1900 mg/L treatment.

There did not appear to be any significant differences between treatments in replicate 3 (Figure 3.14 C). However, the 2530 mg/L treatment was abandoned in the replicate, and if this treatment had followed through to the end of the experiment when animals were dry weighed, it is possible that a significant difference would have been found. The single point with no standard deviations at the 3375 mg/L treatment indicates the dry weight measurement of a single animal.

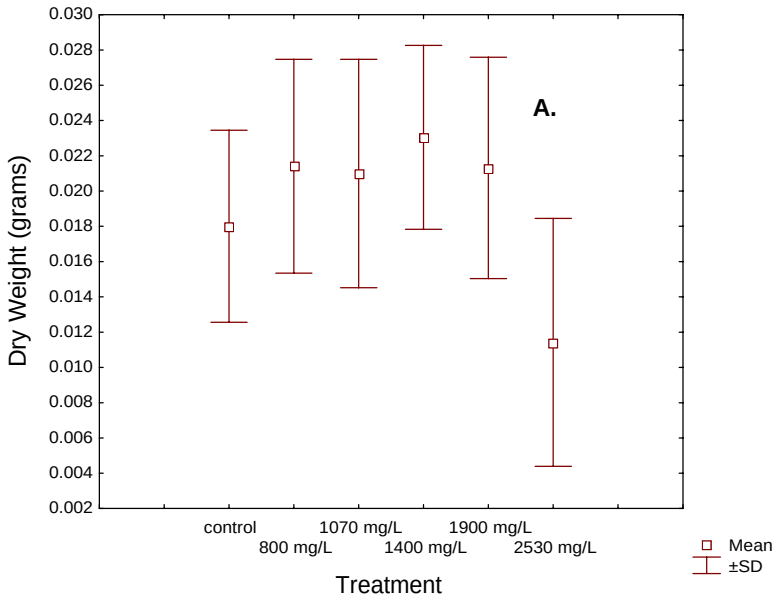


Figure 3.14 Means and standard deviations of growth (dry weight) results for the three replicated concentration ranges from the Na_2SO_4 chronic toxicity experiment (Figure A - replicate 1, Figure B - replicate 2 and Figure C - replicate 3).

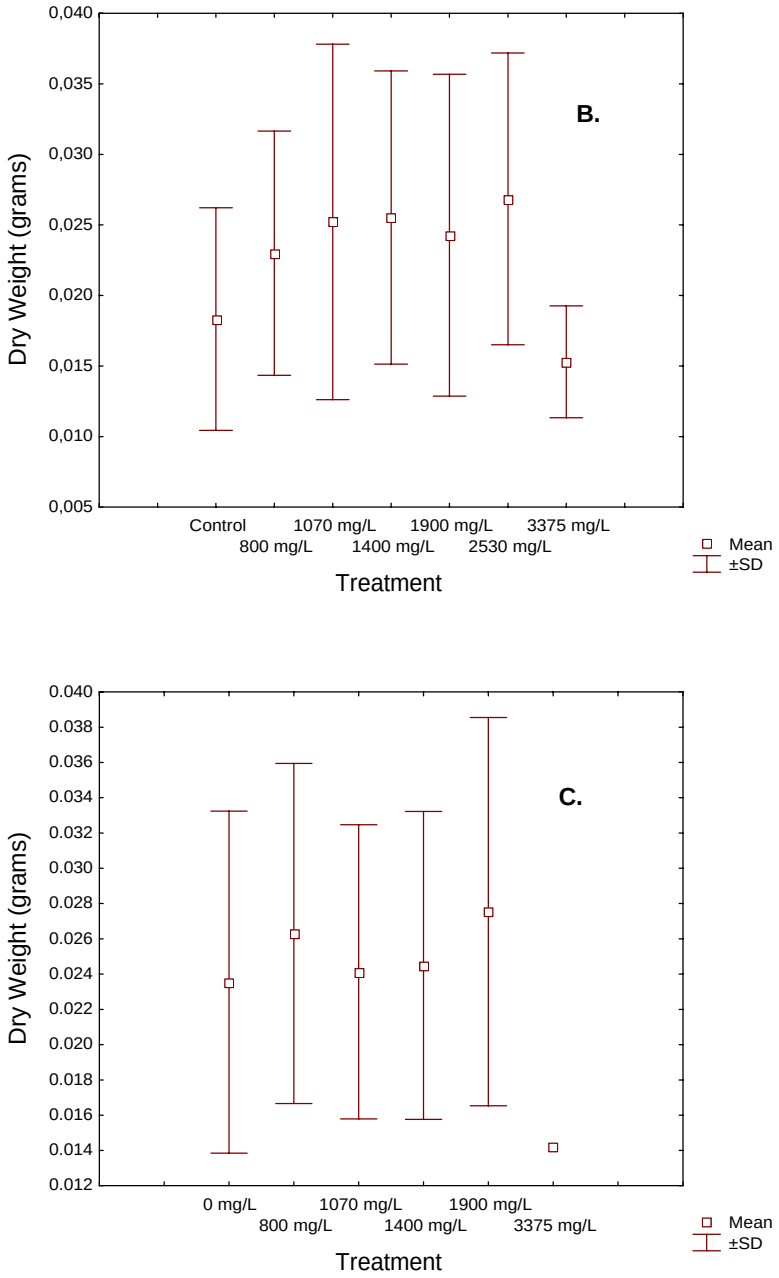


Figure 3.14 continued Means and standard deviations of growth (dry weight) results for the three replicated concentration ranges from the Na_2SO_4 chronic toxicity experiment (Figure A - replicate 1, Figure B - replicate 2 and Figure C - replicate 3).

3.3.2.3 Reproduction

There did not appear to be any negative effect on reproduction by exposure to Na_2SO_4. The proportion of survivors that were gravid actually seems to have increased with increasing Na_2SO_4 exposure (Figure 3.15). Parametric and non – parametric tests however found no significant positive differences (sample sizes depicted in Table 3.4). Significant differences between the 2530 mg/L treatment and the control could not be tested for as all individuals in this treatment in replicate 3 perished before any individuals could reach sexual maturity and treatment data from all three replicates were needed to test for significant differences.

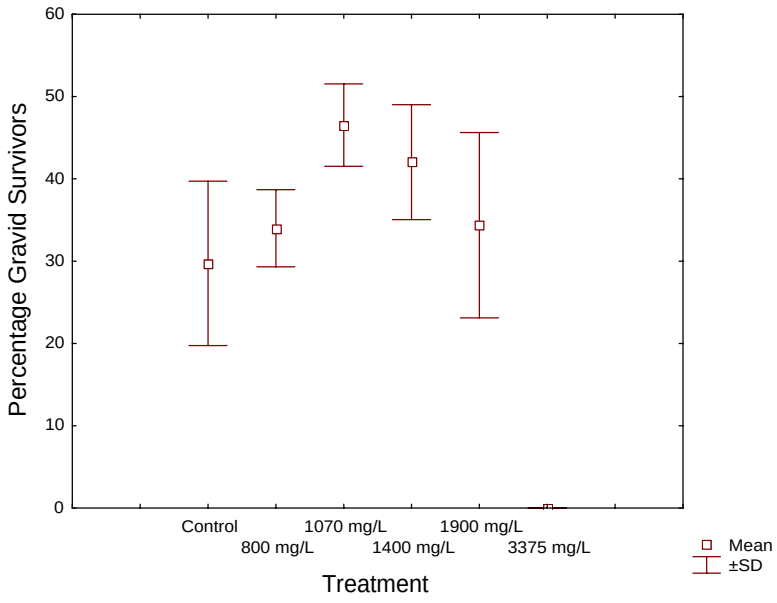


Figure 3.15 Means and standard deviations of the grouped reproduction (percentage gravid survivors) results for the three replicated concentration ranges from the Na_2SO_4 chronic toxicity experiment.

There did not appear to be significant differences among treatments in terms of the number of eggs per female in replicate 1 and 3 (Figure 3.16). This was however tested for replicate 2 (sample sizes depicted in Table 3.4). The data distribution of untransformed data was normal (Shapiro-Wilk $W = 0,94$, $p < 0,05$) but the data was not homogeneous as indicted by the Levene's Test ($p < 0,05$). A non – parametric Kruskal – Wallis found indicated that the 2530 mg/L treatment was significantly

different from all other treatments. The LOEC for these data was the 2530 mg/L treatment while the NOEC was the 1900 mg/L treatment.

The grouped results of all replicated concentration ranges did not seem to show significant differences between treatments (Figure 3.17).

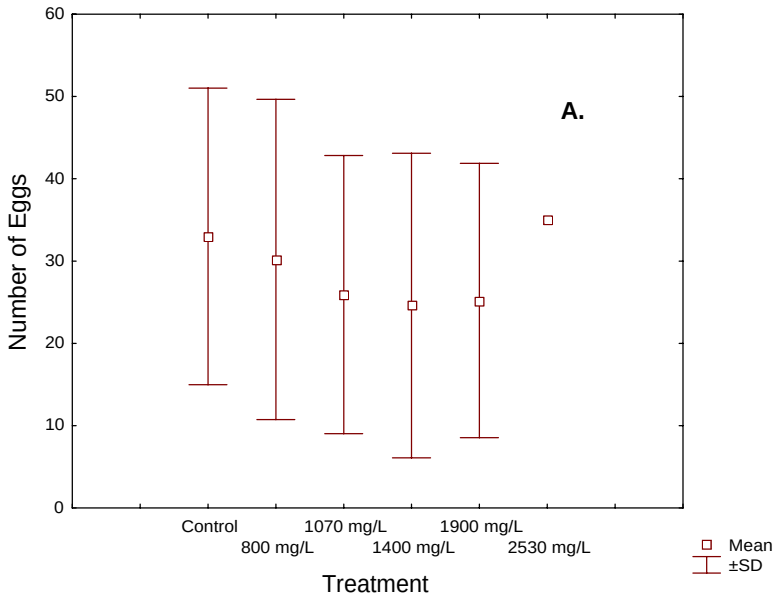


Figure 3.16 Means and standard deviations of reproduction (number of eggs) results for the three replicates from the Na_2SO_4 chronic toxicity experiment (Figure A - replicate 1, Figure B - replicate 2 and Figure C - replicate 3).

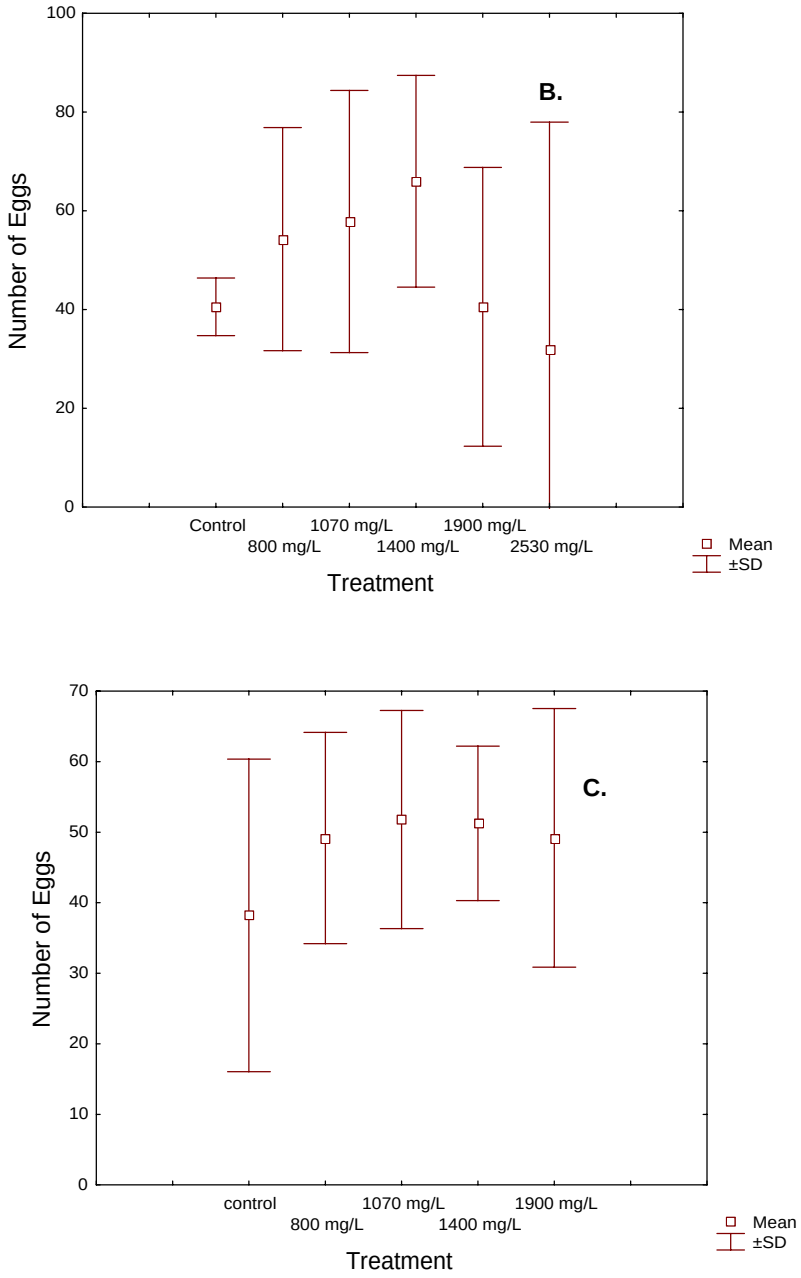


Figure 3.16 continued Means and standard deviations of reproduction (number of eggs) results for the three replicates from the Na_2SO_4 chronic toxicity experiment (Figure A - replicate 1, Figure B - replicate 2 and Figure C - replicate 3).

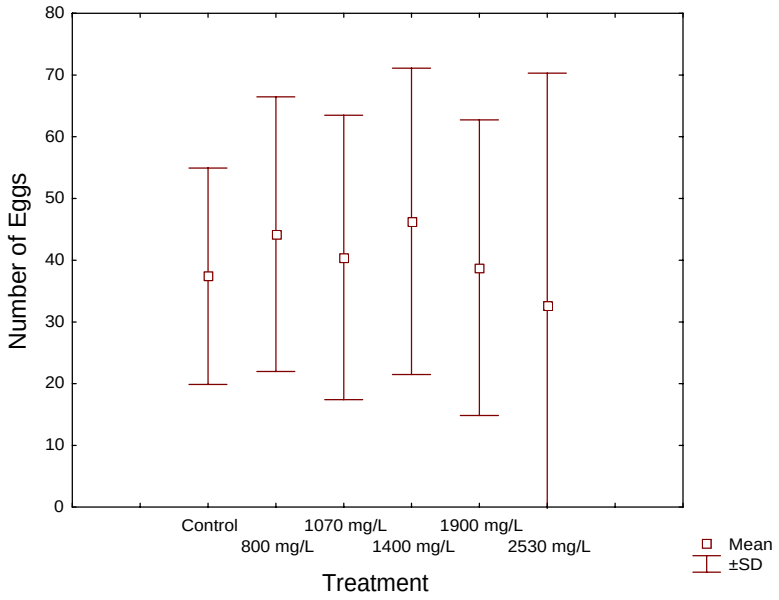


Figure 3.17 Means and standard deviations of reproduction (number of eggs) results for the three replicates grouped from the Na_2SO_4 chronic toxicity experiment.

3.3.4 Comparison of NOECs and LC_x values for mortality

An upper 95 % confidence limit for the LC_{50} value could not be calculated for the mortality results in replicate 1 in the NaCl chronic toxicity test (Table 3.1, Figure 3.4 A). Therefore it could not be determined whether the LC_{50} obtained in replicate 1 was statistically significantly different from the LC_{50} values obtained in replicate 2 and replicate 3. The LC_{50} values obtained in replicate 2 and replicate 3 (Table 3.1, Figure 3.4 B and Figure 3.5) were not statistically significantly different from each other ($p > 0,05$), as determined by the equation given by APHA (1992) cited in Muller and Palmer (2002). Since the LC_{50} values for all three replicate were fairly similar (Table 3.1), it was assumed that the LC_{50} calculated for replicate 1 was not significantly statistically different from those derived from replicate 2 and replicate 3.

The combined mortality results from the NaCl experiment after 80 days duration did not fit the Probit (USEPA, 1993) model. Percent mortality in each replicate was adjusted to take into account the control mortality using Abbot's Formula (Finney, 1971):

$$\%Mortality = 100 * ((T - C)/(100 - C))$$

where T = unadjusted treatment % mortality, C = control % mortality.

The combined mortality results adjusted using Abbot's Formula did not fit the Probit model and therefore the data were analysed using linear regression. Linear regression did not provide a particularly good fit (r^2 = 0,53). Since the mortality results of the third replicate did fit the Probit model and in general mortality results for all three replicates were similair, at least at the LC_{50} level, the LC_5 of the third replicate was used for comparison of the NOEC measured by hypothesis testing. The mortality results from the third replicate of the NaCl chronic toxicity experiment yielded a LC_5 value of 2481 mg/L (Figure 3.18 A). This is similar to the 1900 mg/L NOEC that was determined in the toxicity test via hypothesis testing. The NOEC corresponds to a % mortality of less than 1 (LC_1) determined by the regression (Figure 3.18 A).

There was no statistically significant difference between the mortality LC_{50} values calculated for any of the three replicates of the Na_2SO_4 chronic toxicity test ($p > 0,05$), as determined by the equation given by APHA (1992) cited in Muller and Palmer (2002).

The combined mortality results from the Na_2SO_4 chronic experiment after 80 days duration did fit the Probit (USEPA, 1993) model. The LC_5 value obtained from the Na_2SO_4 mortality results was 1932,6 mg/L. This was close to the experimentally derived NOEC of 1900 mg/L for Na_2SO_4.

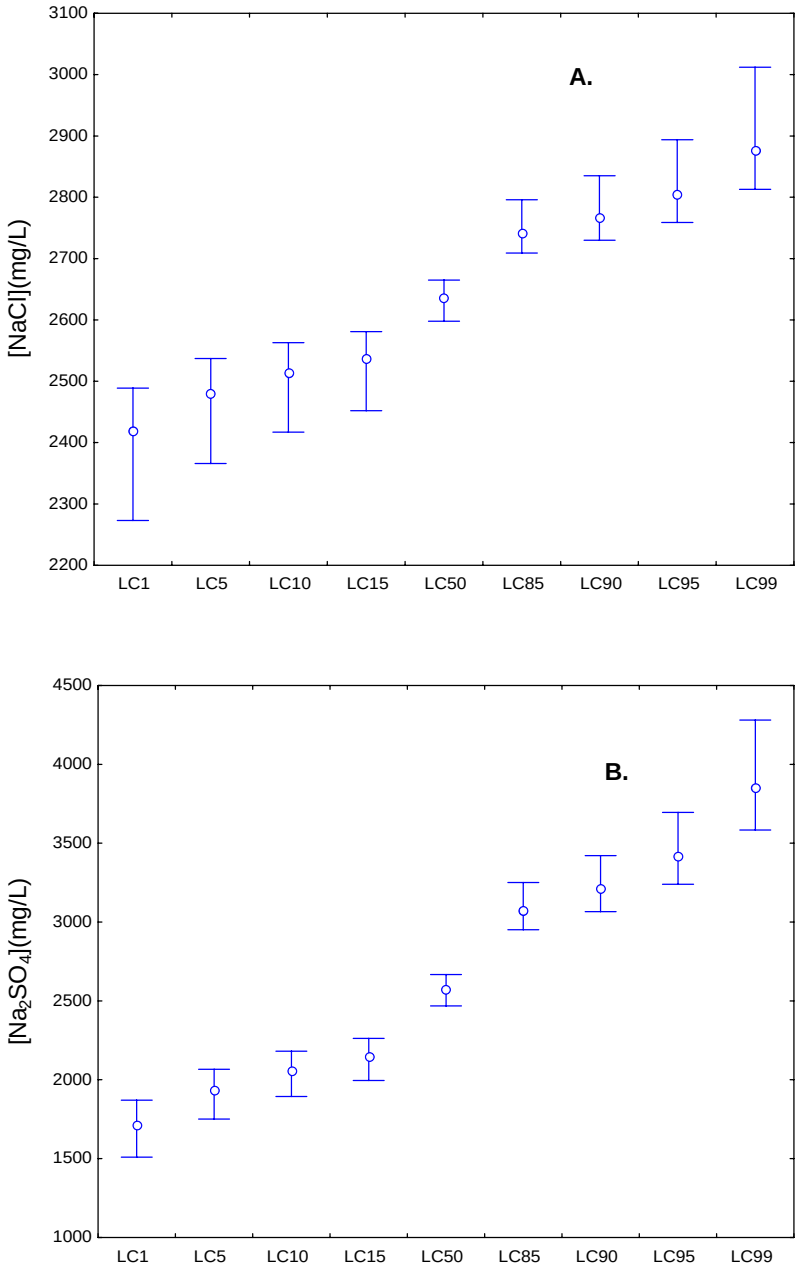


Figure 3.18 A regression model was applied to the combined mortality results for all three replicated concentration ranges for each salt after an 80 days exposure period (Figure A – The regression line for mortality data from the NaCl chronic toxicity test, Figure B – A probit model fitted the mortality data from the Na_2SO_4 chronic toxicity test).

3.3.3 Summary of biological responses

A summary of toxicity measures taken in the chronic toxicity tests for both salts is depicted in Table 3.3.

Table 3.3 A summary of measured toxicity values taken in the chronic toxicity tests for *C.nilotica* exposed to NaCl and Na_2SO_4. 'NA' - Not Applicable. '*' - Could not be calculated.

NaCl							**Na_2SO_4**						
				Value (mg/L)							**Value (mg/L)**		
					95 % confidence limits							**95 % confidence limits**	
Endpoint	**Biological Response**	**Duration**	**Measure**		**Upper**	**Lower**	Endpoint	**Biological Response**	**Duration**	**Measure**		**Upper**	**Lower**
Mortality	Mortality	80 days	LOEC	2700	NA	NA	Mortality	Mortality	80 days	LOEC	2530	NA	NA
Mortality	Mortality	80 days	NOEC	1900	NA	NA	Mortality	Mortality	80 days	NOEC	1900	NA	NA
Mortality	Morality	80 days	LC_{50}	2425	*	1700	Mortality	Morality	80 days	LC_{50}	2531	3800	1900
Mortality	Mortality	80 days	LC_{50}	2490	3200	2120	Mortality	Mortality	80 days	LC_{50}	2803	4000	2200
Mortality	Mortality	80 days	LC_{50}	2637	2665	2598	Mortality	Mortality	80 days	LC_{50}	2670	3500	2000
Growth	Carapace Length	80 days	LOEC	2700	NA	NA	Growth	Carapace Length	39 days	LOEC	3375	NA	NA
Growth	Carapace Length	80 days	NOEC	2500	NA	NA	Growth	Carapace Length	39 days	NOEC	2530	NA	NA
Growth	Carapace Length	80 days	LOEC	2200	NA	NA	Growth	Carapace Length	23 days	LOEC	1900	NA	NA
Growth	Carapace Length	80 days	NOEC	1900	NA	NA	Growth	Carapace Length	23 days	NOEC	1400	NA	NA
Growth	Dry Weight	80 days	LOEC	2200	NA	NA	Growth	Dry Weight	80 days	LOEC	2530	NA	NA
Growth	Dry Weight	80 days	NOEC	1900	NA	NA	Growth	Dry Weight	80 days	NOEC	1900	NA	NA

Table 3.3 continued A summary of measured toxicity values taken in the chronic toxicity tests for *C.nilotica* exposed to NaCl and Na_2SO_4. 'NA' - Not Applicable. '*' - Could not be calculated.

NaCl							**Na_2SO_4**						
				Value (mg/L)							**Value (mg/L)**		
					95 % confidence limits							**95 % confidence limits**	
Endpoint	**Biological Response**	**Duration**	**Measure**		**Upper**	**Lower**	**Endpoint**	**Biological Response**	**Duration**	**Measure**		**Upper**	**Lower**
Reproduction	% Gravid Survivors	80 days	LOEC	2700	NA	NA	Growth	Dry Weight	80 days	LOEC	2530	NA	NA
Reproduction	% Gravid Survivors	80 days	NOEC	1900	NA	NA	Growth	Dry Weight	80 days	NOEC	1900	NA	NA
Mortality	Mortality	80 days	LC_5	2481	2366	2537	Reproduction	Number of Eggs	80 days	LOEC	2530	NA	NA
							Reproduction	Number of Eggs	80 days	NOEC	1900	NA	NA
							Mortality	Mortality	80 days	LC_5	1933	2067	1751

Table 3.4 Sample sizes where significant differences were tested for. Sample size for number of eggs per female = sample size for gravid females per treatment. Number of survivors per treatment = sample size for dry weight.

NaCl				**Na2SO4**			
Replicate	**Treatment (mg/L)**	**Endpoint Measure**	**N**	**Replicate**	**Treatment (mg/L)**	**Endpoint Measure**	**N**
1	0	Total number of test orgamisms	37	1	0	Total number of test orgamisms	38
1	1300	Total number of test orgamisms	33	1	800	Total number of test orgamisms	31
1	1900	Total number of test orgamisms	32	1	1070	Total number of test orgamisms	29
1	2700	Total number of test orgamisms	35	1	1400	Total number of test orgamisms	27
1	3800	Total number of test orgamisms	29	1	1900	Total number of test orgamisms	28
1	5500	Total number of test orgamisms	42	1	2530	Total number of test orgamisms	15
1	7800	Total number of test orgamisms	41	1	3375	Total number of test orgamisms	24
1	11200	Total number of test orgamisms	42	1	4500	Total number of test orgamisms	39
1	16000	Total number of test orgamisms	45	1	6000	Total number of test orgamisms	35
2	0	Total number of test orgamisms	15	2	0	Total number of test orgamisms	40
2	1300	Total number of test orgamisms	26	2	800	Total number of test orgamisms	30
2	1900	Total number of test orgamisms	24	2	1070	Total number of test orgamisms	18
2	2200	Total number of test orgamisms	27	2	1400	Total number of test orgamisms	19
2	2500	Total number of test orgamisms	20	2	1900	Total number of test orgamisms	21
2	2700	Total number of test orgamisms	19	2	2530	Total number of test orgamisms	15

Table 3.4 continued Sample sizes where significant differences were tested for. Sample size for number of eggs per female = sample size for gravid females per treatment. Number of survivors per treatment = sample size for dry weight.

NaCl				**Na2SO4**			
Replicate	**Treatment (mg/L)**	**Endpoint Measure**	**N**	**Replicate**	**Treatment (mg/L)**	**Endpoint Measure**	**N**
2	3300	Total number of test orgamisms	28	2	3375	Total number of test orgamisms	21
2	3800	Total number of test orgamisms	24	2	4500	Total number of test orgamisms	19
2	5500	Total number of test orgamisms	22	2	6000	Total number of test orgamisms	24
3	0	Total number of test orgamisms	18	3	0	Total number of test orgamisms	34
3	1300	Total number of test orgamisms	17	3	800	Total number of test orgamisms	25
3	1900	Total number of test orgamisms	12	3	1070	Total number of test orgamisms	25
3	2200	Total number of test orgamisms	10	3	1400	Total number of test orgamisms	24
3	2500	Total number of test orgamisms	9	3	1900	Total number of test orgamisms	14
3	2700	Total number of test orgamisms	9	3	2530	Total number of test orgamisms	20
3	3300	Total number of test orgamisms	15	3	3375	Total number of test orgamisms	14
3	3800	Total number of test orgamisms	8	3	4500	Total number of test orgamisms	20
3	5500	Total number of test orgamisms	17	3	6000	Total number of test orgamisms	24
2	0	Carapace length	13	1	0	Carapace length	21
2	1300	Carapace length	24	1	800	Carapace length	24
2	1900	Carapace length	22	1	1070	Carapace length	23
2	2200	Carapace length	19	1	1400	Carapace length	23
2	2500	Carapace length	14	1	1900	Carapace length	21
2	2700	Carapace length	6	1	2500	Carapace length	18
3	0	Carapace length	12	1	3375	Carapace length	6
3	1300	Carapace length	15	3	0	Carapace length	20
3	1900	Carapace length	11	3	800	Carapace length	20
3	2200	Carapace length	9	3	1070	Carapace length	19

Table 3.4 continued Sample sizes where significant differences were tested for. Sample size for number of eggs per female = sample size for gravid females per treatment. Number of survivors per treatment = sample size for dry weight.

NaCl				**Na2SO4**			
Replicate	**Treatment (mg/L)**	**Endpoint Measure**	**N**	**Replicate**	**Treatment (mg/L)**	**Endpoint Measure**	**N**
3	2500	Carapace length	7	3	1400	Carapace length	20
3	2700	Carapace length	2	3	1900	Carapace length	15
2	0	Dry weight	13	3	2530	Carapace length	9
2	1300	Dry weight	24	3	3375	Carapace length	1
2	1900	Dry weight	22	1	0	Dry Weight	36
2	2200	Dry weight	22	1	800	Dry Weight	31
2	2500	Dry weight	14	1	1070	Dry Weight	29
2	2700	Dry weight	6	1	1400	Dry Weight	27
1	0	Number of gravid females	5	1	1900	Dry Weight	28
1	1300	Number of gravid females	7	1	2530	Dry Weight	5
1	1900	Number of gravid females	8	2	0	Dry Weight	40
1	2700	Number of gravid females	1	2	800	Dry Weight	28
1	3800	Number of gravid females	0	2	1070	Dry Weight	13
1	5500	Number of gravid females	0	2	1400	Dry Weight	18
1	7800	Number of gravid females	0	2	1900	Dry Weight	20
1	11200	Number of gravid females	0	2	2530	Dry Weight	11
1	16000	Number of gravid females	0	2	3375	Dry Weight	2
2	0	Number of gravid females	5	1	0	Number of gravid females	9
2	1300	Number of gravid females	8	1	800	Number of gravid females	9

Table 3.4 continued Sample sizes where significant differences were tested for. Sample size for number of eggs per female = sample size for gravid females per treatment. Number of survivors per treatment = sample size for dry weight.

NaCl				**Na2SO4**			
Replicate	**Treatment (mg/L)**	**Endpoint Measure**	**N**	**Replicate**	**Treatment (mg/L)**	**Endpoint Measure**	**N**
2	1900	Number of gravid females	6	1	1070	Number of gravid females	15
2	2200	Number of gravid females	4	1	1400	Number of gravid females	10
2	2500	Number of gravid females	4	1	1900	Number of gravid females	6
2	2700	Number of gravid females	0	1	2530	Number of gravid females	1
2	3300	Number of gravid females	0	1	3375	Number of gravid females	0
2	3800	Number of gravid females	0	1	4500	Number of gravid females	0
2	5500	Number of gravid females	0	1	6000	Number of gravid females	0
3	0	Number of gravid females	6	2	0	Number of gravid females	9
3	1300	Number of gravid females	6	2	800	Number of gravid females	11
3	1900	Number of gravid females	1	2	1070	Number of gravid females	6
3	2200	Number of gravid females	0	2	1400	Number of gravid females	9
3	2500	Number of gravid females	0	2	1900	Number of gravid females	8

Table 3.4 continued Sample sizes where significant differences were tested for. Sample size for number of eggs per female = sample size for gravid females per treatment. Number of survivors per treatment = sample size for dry weight.

NaCl				**Na2SO4**			
Replicate	**Treatment (mg/L)**	**Endpoint Measure**	**N**	**Replicate**	**Treatment (mg/L)**	**Endpoint Measure**	**N**
3	2700	Number of gravid females	0	2	2530	Number of gravid females	3
3	3300	Number of gravid females	0	2	3375	Number of gravid females	0
3	3800	Number of gravid females	0	2	4500	Number of gravid females	0
3	5500	Number of gravid females	0	2	6000	Number of gravid females	0
				3	0	Number of gravid females	14
				3	800	Number of gravid females	7
				3	1070	Number of gravid females	10
				3	1400	Number of gravid females	9
				3	1900	Number of gravid females	5
				3	2530	Number of gravid females	0
				3	3375	Number of gravid females	0
				3	4500	Number of gravid females	0
				3	6000	Number of gravid females	0

3.4 Discussion

3.4.1 General discussion

The chronic exposure of *C.nilotica* to NaCl and Na_2SO_4 yielded sub-lethal toxicity data. It is surprising that the NOECs estimated were the same for NaCl and Na_2SO_4,

as one would expect sulphate to be more toxic than chloride, especially since the chloride ion is associated more with natural salinisation (Scherman *et al*., 2003). Jooste and Rossouw's (2002) benchmark values and salt model rank Na_2SO_4 as being more toxic than NaCl. The fact that in most cases, the different biological responses measured produced the same value of NOEC is also surprising. It is reasonable to assume that measured sub-lethal biological responses would produce lower NOEC values than lethality measures, but this was not the case here. Compared to the salinity tolerance of many macroinvertebrates, the experimental NOEC values estimated here for *C.nilotica* were not particularly sensitive (S.Browne *pers.comm.*, 1994). This may be explained by the fact that *C.nilotica* probably had a marine ancestry and these shrimp have been found in waters of elevated salinity and therefore may be tolerant of elevated salinity. Included in *C.nilotica*'s distribution is Lake Sibaya, which is characterised by high salinity (Hart, 1981). Specimens have also been found in a salinity of 4 parts/thousand in the Keiskamma River and 6 parts/thousand in the Bushman's River (Hart, 1983). Unfortunately, since parts/thousand measures are not salt specific, they cannot be compared to the NOEC values determined in the chronic toxicity tests. Looking at the results here, one could even go further and suggest that a low level of dissolved salt is probably beneficial for the growth and development of these shrimp. Mortality results were often less than the control in the lower salt treatments (Figure 3.3 and 3.11), growth seems to be elevated in low salt treatments (Figures 3.7, 3.13, and 3.14) and the same could even be said for reproduction (3.15 and 3.16). Chapman (2002) defines hormesis as a toxicological phenomenon that occurs when a low concentration toxicant treatment has a positive effect on a biological response (mortality, fecundity etc) that is 30 – 60 % higher than that of the control, and the toxicant concentration that has this effect is in the range of ten times less than the NOEC concentration. Dry weight response in replicate 2 of the Na_2SO_4 chronic experiment showed a mean response of 0,019 grams in the control compared to 0,025 grams in the 1070 mg/L treatment. This is 32 % higher dry weight than the control but the 1070 mg/L treatment is not ten times less than the calculated NOEC of 1900 mg/L. This is one of the more pronounced examples from this study of low toxicant concentrations having a positive effect compared to the control, therefore according to Chapman's (2002) definition, no hormetic response was evident in this study.

There were limited numbers of *C.nilotica* individuals available for the chronic toxicity tests, and therefore sample sizes for measurement of significant differences were small in some cases (Table 3.4). Ideally, at least 20 individuals per treatment for carapace measures would have preferable (Muller *pers.comm.*, 2003). In high salt concentration treatments this was very often impossible because very few individuals survived these treatments.

The ANZECC and ARMCANZ (2000) guidelines only used chronic toxicity data, either experimental or extrapolated, to derive trigger values (Warne, 2001). Experimental chronic toxicity data were used to derive high reliability trigger values while acute data were used to derive medium or low reliability trigger values (Warne, 2001). This highlights the importance of chronic toxicity data and in particular experimental chronic toxicity data. A working protocol for using *C.nilotica* in chronic toxicity testing is therefore important in this regard. The protocol provides a method of deriving chronic tolerance information for toxicants for an additional species. This will allow the use of the Species Sensitivity Distribution (SSD) method for more toxicants as there are minimum data requirements for using this method (Warne, 2001), as well provide an extra data point in existing SSDs. The chronic protocol is very important in the South African context as *C.nilotica* is indigenous South Africa, and tolerance information for this species will contribute to the development of future water quality guidelines.

Some of the results of the chronic tests indicate that one may be able to detect significant sub - lethal affects within 20 days. This would appear reasonable for growth (Figures 3.6 B and C, Figure 3.13 C). Mortality definitely begins to show the significant differences between treatments (that persist until the end of the test) by day 20 (Figures 3.3 and 3.11). Therefore significant mortality responses could possibly be measured in a short-term chronic toxicity test of 20 days. Unfortunately, starting off with young juveniles and running a test until day 20, will not allow any individuals to reach female sexual maturity, and therefore reproductive endpoints (number of eggs etc) cannot be measured. It is likely that young juveniles of macroinvertebrates are the most sensitive to toxicants of any life stages in the lifecycle (Hutchinson *et al*., 1998). A study of the relative sensitivities of different life stages of organisms according to toxicity data on the ECETOC database showed that sensitivity of aquatic

macroinvertebrates to toxicants decreased with age (Hutchinson *et al*., 1998). Although there were not enough data for macroinvertebrates, fish larvae appeared to be more sensitive than the embryo stage, probably because eggs are protected by a tegument (Hutchinson *et al*., 1998). This could very well be the case for macroinvertebrates as well. There are acute toxicity data for both *C.nilotica* juveniles and adults exposed to NaCl and Na_2SO_4 on the IWR/UCEWQ database. There are four acute NaCl toxicity tests of 48 hours using *C.nilotica* juveniles of less than seven days old and one NaCl 96 hour acute toxicity tests using *C.nilotica* adults (See Table 2.1 in chapter 2). The 48 hour LC_{50} measures for the 4 toxicity tests using juveniles are: 5955 mg/L, 4450 mg/L, 5979 mg/L and 5487 mg/L with a geometric mean of 5430 mg/L. The 48 hour LC_{50} of the 96 hour *C.nilotica* adult toxicity test is 14693 mg/L. It therefore appears that *C.nilotica* adults are up to three times as tolerant of NaCl as juveniles of less than seven days. There are also four acute Na_2SO_4 toxicity tests of 48 hours using *C.nilotica* juveniles of less than seven days old and one Na_2SO_4 96 hour acute toxicity tests using *C.nilotica* adults (See Table 2.2 in chapter 2). The geometric mean of the juvenile toxicity tests is 6024 mg/L, with individual 48 hour LC_{50} values for the four tests being: 5989 mg/L, 7002 mg/L, 5734 mg/L and 5477 mg/L. In contrast, the 48 hour LC_{50} value of the Na_2SO_4 adult toxicity test is 11194 mg/L. This is almost twice as high as that of the juvenile toxicity test LC_{50}s.

The mortality LC_5 value for NaCl was close to the NOEC estimated via hypothesis testing (Figure 3.18 A). The LC_5 of these data equated to 2480 mg/L. This is slightly less conservative than the calculated NOEC of 1900 mg/L. The mortality data obtained from the Na_2SO_4 chronic experiment did fit the Probit model (Figure 3.18 B). The lethality LC_5 value estimated from the model at 80 days duration was close to the lethality NOEC at 80 days duration, suggesting that using an LC_5 value as indicative of an NOEC proposed by Warne (1998), is justified. However, since the NOEC values being compared to LC_x values are essentially mortality NOEC values, this is hardly surprising. But since most sub-lethal NOEC measures in the chronic toxicity tests were of the same value as the mortality NOECs, the use of an LC_5 as being indicative of an NOEC is justified.

The NOEC concentrations estimated for NaCl and Na_2SO_4 are not statistically significantly different from the control. Biological significance of these estimates

however needs to be considered. It has been shown that the NOEC value for Na_2SO_4 for lethality is equivalent to the death of 5 % of test animals. The long - term implications of death of 5 % of a population of this species in a particular ecosystem is unlikely to be biologically significant. The regression line fitted to the NaCl lethality data produced a lethality percentage of < 1 %. Therefore is is unlikely that the mortality NOEC would have a biologically significant affect. The biological significance of the measured NOECs should additionally be considered in terms of sub-lethal affects for both salts. In the NaCl chronic experiment, mean carapace length measures per replicate in the 1900 mg/L treatment ranged from a negligible amount to 13 % less than mean growth in the control (Figure 3.6 A – C). Mean dry weight measures per replicate in the NaCl chronic test 1900 mg/L treatment ranged from a negligible amount to 36 % less than dry weight in the control (Figure 3.7 A – C). The mean amount of gravid survivors in the 1900 mg/L treatment was 41 % less than in the control (Figure 3.8). The mean number of eggs per replicate in the 1900 mg/L treatment was about 17% less that that of the control (Figure 3.9 A – C). There are therefore some large affects on sub-lethal biological responses by the 1900 mg/L treatment compared to the control for NaCl, despite the fact that the 1900 mg/L treatment was in most cases deemed statistically insignificantly different from the control. The measured NOEC for NaCL may therefore not be biologically insignificant, and the next lowest treatment of 1300 mg/L may be more protective as an NOEC. This however needs to be investigated further. The mean carapace length per replicate for the Na_2SO_4 chronic test 1900 mg/L treatment ranged from a negligible amount to 8 % more than the control (Figure 3.13 A – C). Mean dry weight measured per replicate in the 1900 mg/L treatment ranged from 16 to 33 % more that that measured in the control (Figure 3.14 A – C). The mean number of gravid survivors in the 1900 mg/L treatment was 26% less than that of the control (Figure 3.15) while the mean number of eggs per replicate for the 1900 mg/L treatment ranged from an insignificant amount to 32% more than that of the control (Figure 3.16). The measured NOEC for Na_2SO_4 is therefore unlikely to be negatively biologically significantly different from the control.

Jones (1981) found a seasonal shift in salinity tolerance in the estuarine crab *Helice crassa* related to temperature. The tests run with *C.nilotica* were done under controlled conditions with particular emphasis on controlled water and air temperature

and controlled light. Therefore the assumption has been made that there has been no seasonal shift in tolerance, although tests have been running throughout the year. In addition, the laboratory cultures of *C.nilotica* have been running for 6 years under controlled temperature and light conditions and seasonal responses are probably less pronounced than would occur in wild populations.

Generally mortality in the NaCl chronic experiment stabilized after 20 days (Figure 3.3). This is consistent with the theory behind Two- Step Linear Regression (Mayer *et al*., 1994) and Multi-Factor Probit Analysis (Lee *et al*., 1995). These methods attempt to predict time independent tolerance of organisms to a particular toxicant. Mortality in the Na_2SO_4 chronic experiment however did not show obvious stabilisation.

In many cases, the LOEC values measured in the chronic toxicity tests, for both NaCl and Na_2SO_4, are greater than the LC_{50} values measured for the salts via regression analysis (Table 3.3). It is evident that for both salts, the NOEC represents a threshold where all concentrations lower or equal to the NOEC cause very little mortality and mortality sharply rises at all concentrations higher than the NOEC. The fact that the mortality NOEC is equivalent to the LC_5 and less than the LC_1 for Na_2SO_4 and NaCl respectively and the LOEC is greater than the LC_{50} for NaCl and similar to the LC_{50} for Na_2SO_4, testifies to the fact that *C.nilotica* is subject to a very sharply defined threshold of toxicity to both salts. For NaCl the LOEC is greater than the NOEC by 800 mg/L while the LOEC for Na_2SO_4 is greater than the NOEC by 630 mg/L. Considering the concentration ranges used in the tests these are not great differences.

3.4.2 Caridina nilotica as a chronic test animal

The results seem to indicate that *C.nilotica* is fairly tolerant of elevated salinities. The NOEC values obtained for *C.nilotica* would probably not be protective of most other aquatic macroinvertebrates at a chronic exposure duration. Salinity tolerance information for this species are still important for setting salinity water quality guidelines in South Africa, as this is an indigenous species and new methods of setting guidelines, such as Species Sensitivity Distributions, use all available tolerance data, and not just the most sensitive tolerance data (Warne, 1998).

C.nilotica may be more sensitive to toxicants other than the salts tested here. Sloof (1983), in an investigation on the comparative sensitivity of 22 freshwater species to 15 chemical compounds, found that crustaceans were among the most sensitive macroinvertebrates. The IWR/UCEWQ toxicity database contains the results of acute toxicity testing where this species was exposed to cadmium chloride. The LC_{50}s ranged from 0,05 – 0,09 mg/L. If compared to the LC_{50} values of approximately 0,15 mg/L for *Daphnia pulex* (a standard toxicity test species) on the database, *C.nilotica* appears to be sensitive for this chemical at least, although this needs to be confirmed for other toxicants.

C.nilotica is readily reared under laboratory conditions. The biology and life history of the species has also been researched (Hart, 1980; Hart 1981; Hart, 1982). Equipment for rearing this species is relatively inexpensive. Food suitable for this species is also readily available and inexpensive. Egg to egg lifecycle takes approximately 3 months, which is long enough for a comprehensive chronic toxicity test but short enough to not pose logistic problems. This species is also indigenous to South Africa. Individuals are fairly easy to handle at a mature age with full-grown animals reaching up to 2½ cm in length. Even young neonates are relatively easy to handle as they are visible with the naked eye and are readily transferable from one container to another using a pipette. One point of apparent concern is the high natural attrition rate of young neonates under 14 days old. However, using slightly older neonates as a starting point within a chronic toxicity test bypasses this problem. Using reproduction as an endpoint with this species in a toxicity test requires careful thought and planning. These shrimps are sexed according to the presence or absence of the appendix masculina on the excised 2nd pleopods, but reliable sex determination of living individuals is not possible (Hart, 1980). Therefore at the start of an experiment, it is impossible to control the ratio of sexes in the treatments. It is likely that all individuals in a treatment may be of the same sex. The sex of these shrimp may be related to size, with individuals changing from male to female when they reach a certain size (Okuthe *et al*., 2004). If individuals of the same age and size are used at the start of an experiment, and growth of all individuals are fairly standard, most individuals will be of the same sex at any particular time and reproduction will not be possible whereas a treatment where there is more variability in regards to the size of the individuals will probably contain greater heterogeneity in the sex of the

individuals. It is also possible that larger individuals may be more tolerant to a certain stressor. In this case all smaller individuals in a treatment may perish effectively leaving survivors in larger size categories. All the survivors may then be female, which would exclude reproduction. A suitable reproductive endpoint that would bypass this problem would be mean number of eggs per female.

Another issue that needs to be considered when using *C.nilotica* as a toxicity test organism, is that individuals have a habit of jumping out of the experimental vessels. In this test, vessels were covered with cling wrap, which effectively prevented this. Unfortunately cling wrap tends to lose its effectiveness over time as corners of the covering start bunching up and small openings appear. The cling wrap coverings were replaced regularly in these experiments to prevent this. It is recommended that something more permanent be used to cover the vessels such as sheets of glass.

All points considered, *C.nilotica* is an excellent indigenous organism to use for chronic toxicity tests where growth as a biological response is measured. The potential use of this organism in short-term partial lifecycle chronic toxicity tests needs to be investigated.

Chapter 4:Validation of acute to chronic toxicity data extrapolation techniques

4.1 Introduction

Freshwater resources in South African are coming under increasing pressure. A growing population and associated increased domestic, agricultural and industrial needs are leading to increased exploitation of freshwater resources (Walmsley *et al*., 1999). Subsequently, much of South Africa has been recognised as water stressed (Alcamo and Henrichs, 2002), and all major rivers are currently being regulated (Walmsley *et al*., 1999). Associated with increased exploitation of water resources is the deterioration of water quality. Surface waters are becoming polluted by industrial effluent, sewage, irrigation return flows, litter and acid mine drainage, with many rivers experiencing increased nutrient and salinity levels (Ormerod, 1999; Walmsley *et al*., 1999).

In order to protect freshwater resources in South Africa, the South African National Water Act (No. 36 of 1998) stipulated the need for an ecological Reserve. The ecological Reserve defines the water required in terms of both quantity and quality, to sustain the health of aquatic ecosystems while allowing sustainable use of water resources. The water quality needs of the ecological Reserve are defined by proposed generic boundary values that are linked to ecosystem health classes (Jooste and Rossouw, 2002). South Africa currently has water quality guidelines for aquatic ecosystems in place in the form of Volume 7 of the South African Water Quality Guidelines (DWAF, 1996). Water quality guidelines are numerical concentration units or narrative statements for substances recommended to support and maintain a designated water use or ecosystem condition (ANZECC, 1992).

The benchmarks for the ecological Reserve (Jooste and Rossouw, 2002) give guidance on the tolerance limits of aquatic fauna and flora to the various toxicants that they may be exposed to. Aquatic toxicity testing forms an integral part of determining what those tolerance limits are.

Aquatic toxicity testing falls into two broad categories according to exposure time, namely acute and chronic toxicity testing. Acute toxicity testing usually measures exposure over 4 days or less and survival is usually the biological response measured (Rand, 1995). In the compilation of the Australian and New Zealand Water Quality Guidelines for toxicants (ANZECC and ARMCANZ, 2000), acute tests were defined for multi-celled organisms as tests where exposure time lasted between 24 and 96 hours (Warne, 2001). Chronic tests were defined as tests that have an exposure time that is longer than 96 hours (Warne, 2001). In contrast, Jooste and Rossouw (2002) took a different approach in terms of categorising toxicological data, with all lethality data used to derive a lethality menchmark and all sub-lethal data used to derive a sub-lethality benchmark. In this thesis, acute toxicity tests have been defined as tests with an exposure duration of 96 hours or less. Chronic tests have been defined as toxicity tests with a duration exceding 96 hours. Since chronic toxicity data give a more environmentally realistic indication of tolerance limits of aquatic biota to long-term exposure to toxicants, chronic data are regarded as more relevant for the setting of water quality guidelines.

The South African Water Quality Guidelines (DWAF, 1996) are based predominantly on international aquatic toxicity data, because of a lack of knowledge of the tolerance limits of indigenous aquatic species (Roux *et al*., 1996). In addition, there is a lack of chronic aquatic eco-toxicity data internationally because of the difficulty and expense of running chronic toxicity tests. This is why Acute to Chronic Ratios ACRs were used to extrapolate chronic toxicity data in the compilation of the ANZECC and ARMCANZ (2000) trigger values (Warne, 2001).

Apart from ACRs that have been used to generate predicted chronic tolerance data from acute toxicity data (Warne, 1998), various statistical acute to chronic extrapolation methods have been proposed. Two-Step Linear Regression (LRA) (Mayer *et al*., 1994) and Multi-Factor Probit Analysis (MPA) (Lee *et al*., 1995) are two extrapolation methods that take duration of exposure to toxicants into account, and both have been validated for toxicity tests using fish, by the respective authors. The possible use of these extrapolation methods provides a cheaper and more rapid alternative to experimental chronic toxicity testing. The generation of chronic toxicity data for indigenous aquatic macroinvertebrates using acute to chronic extrapolation

could provide information with which the South African Water Quality Guidelines (DWAF, 1996) and Jooste and Rossouw's (2002) benchmarks could be refined to be more representative of South African biota.

Although MPA and LRA have been validated for fish, macroinvertebrates make up a large proportion of aquatic fauna, and therefore acute to chronic extrapolation methods need to be validated for macroinvertebrates. Validation needs to occur in two respects. Acute to chronic extrapolation methods need to consistently generate predicted chronic tolerance data that is as conservative or more conservative than tolerance data produced by experimental chronic toxicity testing. In this way, one can be sure that aquatic biota are sufficiently protected by guidelines produced using extrapolated chronic data. It is possible that acute to chronic extrapolation methods may consistently produce chronic tolerance data that are too conservative, leading to guidelines that are restrictively and unrealistically conservative and preventing the sustainable use of water resources. Therefore acute to chronic extrapolations need to be reliable and the predicted chronic tolerance data they produce should be in the range of chronic tolerances measured in experimental chronic toxicity testing. It must be noted that acute to chronic extrapolation aims to predict the chronic response that would occur experimentally from the results of acute toxicity tests, and makes no provision for environmental reality. The process of generating the ANZECC and ARMCANZ (2000) trigger values preferred the use of safety factors as a precautionary approach to considering environmental realism (Warne, 2001).

Chapter 2 showed that it is statistically possible to extrapolate chronic data from acute data. In Chapter 2, ACRs, LRA and MPA were assessed in terms of the numerical conservativeness of predicted chronic tolerance data they produced. Chapter 3 contains details of chronic toxicity testing performed on *Caridina nilotica*, a freshwater shrimp indigenous to South African freshwaters. The data presently in Chapter 2 and Chapter 3 enable the fulfilment of the following aims of this chapter:

1. To assess the level of protection offered by MPA and LRA in terms of the experimental chronic toxicity test data available.
2. To assess the accuracy of MPA and LRA in relation to available experimental chronic toxicity test data available.

3. To compare acute to chronic extrapolation methods on the basis of the PC95 values produced by Species Sensitivity Distributions on extrapolated chronic data and to compare these values with the boundary values produced by Jooste and Rossouw (2002).

ACRs will not be assessed in Aim 2 and 3 as the chronic data used to generate the ACRs used in chapter 2 and the chronic data used in this chapter to validate the extrapolation methods are the same data and due to the nature of the ACR, validation of the results of the ACRs using these chronic data would be meaningless. Future assessments of ACRs in the context of the data presented here could use ACRs derived from independent data.

4.2 Materials and methods

In this chapter, validation of chronic toxicity data occurred on a single species level. Validation of the extrapolated chronic toxicity data provided by LRA and MPA can occur on two levels with the available experimental chronic toxicity data. Experimental chronic toxicity data are available as NOEC values for *C.nilotica* exposed to NaCl and Na_2SO_4 (see Chapter 3). There are also short-term chronic toxicity data on the IWR/UCEWQ database for which there are three replicates. It is possible to calculate a NOEC for these tests, provided that control mortalities were acceptable in all three replicates. Short-term chronic toxicity data also provide chronic toxicity measures in the form of LC_{50}s.

4.2.1 Validation of extrapolated chronic data using short - term experimentally derived chronic LC_{50} values

Although Mayer *et al*. (1994) and Lee *et al*. (1995) recommended low percentile effect extrapolated data in the range of $LC_{0,01}$ – LC_{10} to be equivalent to a PNOEC for LRA and MPA respectively, LRA and MPA also generate time-independent (asymptotic) LC_{50} values. Since it has been found that mortality in toxicity tests generally stabilise within 10 days (Jooste and Rossouw, 2002; Chapter 2 Figures 2.19 – 2.20), extrapolated LC_{50} values generated by LRA and MPA can be validated by LC_{50} values from short-term chronic toxicity tests of 10 days duration (see Tables 2.1 and 2.2 in Chapter 2). Asymptotic/incipient LC_{50} values were extrapolated from 96-

hour acute toxicity data from the IWR/UCEWQ toxicity database (see Tables 2.1 and 2.2 in Chapter 2) using LRA and MPA in these cases with equivalent (same species and toxicant) short-term chronic data. These extrapolated LC_{50} values were compared for accuracy and protectiveness to equivalent short-term chronic LC_{50} values. Ratios of extrapolated chronic data to experimental chronic data were calculated to assess the relative protectiveness of the extrapolated chronic data produced by LRA and MPA. The LC_{50} value was chosen for comparison because of the model independence and high confidence of this measure as compared to very low or very high LC_x values (Moore and Caux, 1997; Isnard *et al*., 2001; Clarke *et al*., 2002). Where there were more than one short-term chronic LC_{50} value available for a particular species, the geometric mean of the LC_{50}s was compared to the extrapolated LC_{50} value. The geometric mean was used to lessen the effect of skewed data (Warne *pers.comm.*, 1994).

4.2.2 Validation of extrapolated chronic data using experimentally derived chronic NOEC values

There are experimental NOEC values available for the shrimp *C.nilotica,* exposed to NaCl and Na_2SO_4 (see Chapter 3) and from the mayfly *E.elegans,* exposed to NaCl (see Chapter 3 Figure 2.12). The extrapolated chronic LC_1 value for a particular species exposed to NaCl or Na_2SO_4 produced by LRA and MPA was chosen to represent the PNOEC. This is the modal LC_x value in the range of $LC_{0,01}$, $LC_{0,1}$, LC_1, LC_5 and LC_{10} recommended by Mayer *et al*. (1994) and Lee *et al*. (1995) to represent the PNOEC. It was decided to additionally use the extrapolated LC_{50} /5 produced by LRA and MPA as a possible PNOEC. The LC_{50} value is model independent (Moore and Caux, 1997) and has the highest associated confidence intervals (Clarke *et al*., 2002). The LC_{50} values of chronic tests for metal toxicants were divided by an application factor of 5 in the compilation of the Australian and New Zealand Water Quality Guidelines for Toxicants (ANZECC and ARMCANZ, 2000; Warne, 2001). This was only applied to metal toxicant as chronic data for most other toxicants were represented by NOEC values (Warne, 2001). The PNOECs produced by LRA and MPA were compared to experimental NOECs for the same species and toxicant and assessed in terms of protectiveness and accuracy. Ratios of extrapolated chronic data to experimental chronic data were calculated to assess the relative protectiveness of

the extrapolated chronic data produced by LRA and MPA. All available applicable acute (96 hour) and short-term chronic (10 day) data on the IWR/UCEWQ database were used to obtain the PNOECs. The comparison of PNOECs attained through extrapolation using acute and short-term chronic data with NOECs attained using short-term chronic data (as is the case with *E.elegans* exposed to NaCl) is justified, as MPA and LRA incorporate the time of exposure into calculation of predicted chronic response.

4.2.3 Comparison of PC95 values produced using extrapolated chronic toxicity data

The process of generating the ANZECC and ARMCANZ (2000) trigger values utilised Species Sensitivity Distributions (SSDs) (Warne, 2001). Chronic data extrapolated from acute toxicity data using Acute to Chronic Ratios (ACRs) were processed by SSDs to generate medium reliability trigger values (Warne, 2001). Before the SSD method could be used to generate the trigger values, the available toxicity data had to represent at least 5 species and at least 4 major taxonomic groups (e.g. fish, crustaceans, insects, algae etc).

In this chapter, the extrapolated chronic data produced by each of the acute to chronic extrapolation methods, i.e. the ACR, the LRA and the MPA methods, were used in Species Sensitivity Distributions using the BurrliOZ software (CSIRO, 2000), which uses the Burr Type III family of species sensitivity distributions (Warne, 2001). PC95 values (the Protection Concentration that protects 95 % of species) produced by the SSDs were compared to the boundaries between the Good and Excellent river health classes for NaCl and Na_2SO_4 proposed by Jooste and Rossouw (2002). The extrapolated chronic data available in this study represent at most 4 major taxonomic groups (i.e. insects, a gastropod, a flatworm and a crustacean), although the range of taxonomic groups represented in extrapolated chronic data for any particular extrapolation method may be less than 4. The PC95 values produced here are not fully representative of the wide range of taxonomic groups comprising aquatic biota, and therefore do not represent comprehensive water quality guidelines. The PC95 values produced using the extrapolated chronic toxicity data were discussed in relation to Jooste and Rossouw's (2002) boundary values. There are no salt specific salinity

guidelines within the ANZECC and ARMCANZ (2000) trigger values, and therefore no comparison between PC95 values from extrapolated data and trigger values given in the ANZECC and ARMCANZ (2000) trigger values could be made.

4.3 Results

4.3.1 Validation of extrapolated chronic data using experimentally derived short - term chronic LC_{50} values

Table 4.1 lists the results of time-independent LC_{50} values produced by LRA and MPA from 96 hour acute toxicity tests and equivalent LC_{50} values from experimental short-term chronic toxicity tests. LC_{50} values produced by MPA are generally much less conservative than those of the experimental short-term tests. The MPA LC_{50}: short –term chronic LC_{50} ratio ranges from 2 – 35. LC_{50} values produced by LRA are more conservative than the equivalent experimental short –term chronic LC_{50} values in 4 out of 6 cases. In 2 cases, the extrapolated LC_{50} values are slightly less conservative than the equivalent experimental short-term chronic LC_{50}s. The LRA LC_{50}: short –term chronic LC_{50} ratio ranges from 0,27 – 1,37.

Table 4.1. Comparison of asymptotic/incipient LC_{50} values produced by MPA and LRA from 96 hour acute toxicity data with equivalent LC_{50} values taken from short-term chronic toxicity tests.

Species	**Salt**	**Geometric mean short-term chronic LC_{50}(mg/L)**	**LRA LC_{50}**		**Ratio**	**MPA LC_{50}**			**Ratio**
			mg/L	**R^2**		**mg/L**	**chi^2**	**tabulated chi^2**	
Flatworm sp.	Na_2SO_4	8529	2276	0,91	0,27	74268	38,8	79,3	8,71
A.barnardi	NaCl	3186	4362	0,51	1,37	8394	122,7	226	2,63
A.peringueyi	NaCl	2947	1461	0,08	0,50	103145	264,7	406,2	35,00
A.sylvatica	NaCl	4502	5970	0,65	1,33	9899	104,3	162	2,20
E.elegans	NaCl	5480	2339	0,31	0,43	37055	40,33	115,2	6,76
T.tinctus	NaCl	651	224	0,89	0,34	1323	50,5	166	2,03

4.3.2 Validation of extrapolated chronic data using experimentally derived chronic NOEC values

Table 4.2 lists the values of LC_1 and $LC_{50}/5$ PNOECs produced using MPA and LRA and the equivalent NOECs produced in experimental chronic and short – term chronic toxicity tests. PNOECs produced using the LRA LC_1s were more conservative than equivalent experimental NOECs in all cases. Unfortunately, in one case, the LC_1 value produced was less than zero and therefore could not be used. In the two cases where this extrapolation method did produce data, the LRA LC_1: equivalent experimental NOEC ratio ranged from 0,19 – 0,84. LRA LC_{50} /5 produced usable data in all three cases. PNOECs using this method were more conservative than equivalent NOECs in all cases. The LRA LC_{50} /5: equivalent experimental NOEC ratio ranged from 0,1 – 0,95. The MPA LC_1 method produced usable data in two out of three cases. The MPA LC_1: equivalent experimental NOEC ratio ranged from 0,06 – 0,3. MPA LC_{50}s /5 produced usable data in all three cases but PNOECs using this method were more conservative than equivalent experimental NOECs in only two out of three cases. The MPA LC_{50} /5: equivalent experimental NOEC ratio ranged from 0,3 – 3,2.

All extrapolation methods excluding the MPA LC_{50} /5 method, produced PNOEC values that were more conservative than the experimental NOEC values. Therefore the LRA LC_1, the LRA LC_{50} / 5 and the MPA LC_1 extrapolation methods were all validated in terms of the protectiveness of the predictive chronic data they produced given the limited experimental chronic toxicity data available for validation. The LRA LC_{50} / 5 method seemed to produce the most accurate PNOEC values in relation to the limited experimental NOEC values available. The LRA LC_{50} /5 method also produced usable PNOEC values in all cases. Interestingly, PNOECs produced by the extrapolation methods portrayed Na_2SO_4 as being more toxic to *C.nilotica* than NaCl. Yet the experimental chronic toxicity tests seem to indicate that the two salts have the same degree of toxicity to *C.nilotica*. The acute 96 hour toxicity tests for *C.nilotica* on the IWR/UCEWQ toxicity database indicate that Na_2SO_4 is not statistically more toxic than NaCl (Table 2.1 and 2.2 in Chapter 2) over a 96 hour exposure period ($p > 0,05$). The difference in toxicity between NaCl and Na_2SO_4 as indicated by the extrapolated PNOEC values must therefore be as a consequence of the presence of

short-term chronic toxicity data for Na_2SO_4 and the absence of this type of data for NaCl.

Table 4.2. Comparison of LC_1 and $LC_{50}/5$ values produced by MPA and LRA with equivalent (same species and toxicant) NOEC values taken from chronic and short – term chronic toxicity tests.

		NOEC	**LRA PNOEC (LC_1)**			**LRA PNOEC ($LC_{50}/5$)**			**MPA PNOEC (LC_1)**		**MPA PNOEC ($LC_{50}/5$)**		**MPA Goodness of Fit**	
Species	**Salt**	**Mg/L**	**mg/L**	**R^2**	**Ratio**	**mg/L**	**R^2**	**Ratio**	**mg/L**	**Ratio**	**mg/L**	**Ratio**	**Chi^2**	**Tabulated Chi^2**
C.nilotica	NaCl	1900	1599	0,75	0,842	1800	0,52	0,95	571	0,3	768	0,404	180,8	254
E.elegans	NaCl	3000	560	0,52	0,187	258	0,27	0,09	182	0,06	9700	3,233	1060,8	1163
C.nilotica	Na_2SO_4	1900	< 0			763	0,67	0,4	< 0		607	0,32	273	761

4.3.3 Comparison of PC95 values produced using extrapolated chronic toxicity data

Table 4.3. Comparison of PC95 values calculated using chronic toxicity data produced by the ACR, LRA (Mayer *et al*., 1994) and MPA (Lee *et al*., 1995) acute - chronic extrapolation methods.

	NaCl			**Na_2SO_4**		
Method	**Number of Taxonomic Groups**	**Number of Species**	**PC95 (mg/L)**	**Number of Taxonomic Groups**	**Number of Species**	**PC95 (mg/L)**
ACR	3	16	512	4	16	258
LRA $LC_{50}/5$	3	12	118	4	12	61
MPA $LC_{50}/5$	2	13	102	4	13	93
LRA LC_1	3	8	48	1	6	167
MPA LC_1	2	7	133	4	8	457
Jooste and Rossouw (2002) sub-lethality boundary values	**45**			**20**		

Table 4.3 shows the results of the Species Sensitivity Distributions, using chronic data produced by the ACR, LRA and MPA acute to chronic extrapolation methods. The ACR and MPA LC_1 methods produced the least conservative PC95 values for both salts. The LRA LC_1 method produced a conservative PC95 value for NaCl but one of the least conservative PC95 values for Na_2SO_4. The LRA $LC_{50}/5$ and MPA $LC_{50}/5$ methods produced relatively conservative PC95 values for both salts. All PC95 values produced were significantly less conservative than the benchmarks proposed by Jooste and Rossouw (2002), except for the LRA LC_1 PC95 for NaCl.

4.4 Discussion

4.4.1 Determination of the most accurate extrapolation technique

Among the LRA and MPA extrapolation techniques, LRA was both the most conservative and the most accurate when comparing asymptotic LC_{50} values with experimental short-term chronic LC_{50}s. In most cases, the predicted LC_{50} values were more conservative than the experimental LC_{50}s, and the two predicted LC_{50}s that were higher, were only slightly less conservative than the experimental LC_{50}s. MPA

appears to produce unrealistically high asymptotic LC_{50} values. Lee *et al*. (1995) however, recommended the results of MPA over LRA if both methods gave usable results. It certainly seems that MPA gives the most conservative PNOEC values using LC_x values in the range of 0,01 – 10, as the authors suggest. The conservativeness of the MPA method may however not be appropriate as data extrapolated using MPA may be environmentally or sustainably relevant. In Chapter 2, LC_1s derived using MPA were the most conservative of the PNOECs generated by extrapolation techniques. Unfortunately, this method often gives unusable negative numbers. Asymptotic LC_1s generated through LRA also appear subject to the same problem. When comparing PNOECs generated by the extrapolation techniques with equivalent experimental NOECs, LRA seems the most accurate when using the asymptotic $LC_{50}/5$ method. Although the values produced via this method are not as conservative as the extrapolated LRA and MPA LC_1 methods, all PNOECs produced using this method were more conservative than the equivalent experimental NOECs. This method also produced more usable PNOEC values, in this chapter and in Chapter 2. Extrapolated chronic data from the LRA $LC_{50}/5$ method produced the most conservative PC95 values when considering both salts (Table 4.3). The PC95 values produced were much less conservative than the boundary values proposed by Jooste and Rossouw (2002). This may be as a result of several factors. The extrapolated chronic data used in this chapter represent a limited range of taxonomic groups, and may therefore not realistically represent the diversity of aquatic biota sensitive to salts. In addition, the species represented in the extrapolated chronic data may fall into the category of species with medium to high tolerance of salts, with few or no species that are sensitive to salinity. The boundary values proposed by Jooste and Rossouw (2002) may also be too conservative. Jooste and Rossouw (2002) used toxicity data sourced from international toxicity databases, and therefore their proposed boundary values protect many species that are not found in South African freshwater ecosystems. Jooste and Rossouw (2002) used a definition of chronic toxicity data based on sub-lethal endpoints while the definition of chronic toxicity data used in this study was more dependant on exposure duration. Therefore it is possible that Jooste and Rossouw (2002) used more conservative chronic toxicity data to derive the sub-lethal benchmarks then they would have if lethal endpoint chronic data had been considered.

The LC_{50} value is model independent, unlike low LC_x values (Moore and Caux, 1997; Isnard *et al*., 2001) and this measure has the highest associated confidence (Clarke *et al*., 2002). The use of a safety factor of 5 is a trade off because, although the LC_{50} is the best value to extrapolate, 50 % effect is much too high for an NOEC. In fact, Moore and Caux (1997), in a study of pesticide toxicity test data, found that 77 % of NOECs exerted effects between 10 and 30 %. The chronic toxicity test using *C.nilotica* exposed to Na_2SO_4 outlined in Chapter 3, generated an NOEC that had an equivalent 5 % effect using regression analysis. Isnard *et al*. (2001) in a comparison of NOEC and EC_x values produced in chronic toxicity testing using hypothesis and regression statistics respectively, found that the median EC_{50}/NOEC ratio was 2,3. A safety factor of 5 is therefore likely to protective. This safety factor was only applied to metal toxicants in the derivation of the ANZECC and ARMCANZ (2000) guidelines because other toxicants had sufficient NOEC data (Warne, 2001). The value of the safety factor was determined by the expert opinion of Dr John Chapman (NSW EPA) and was based on examining the data collated to derive the Trigger Values in the ANZECC and ARMCANZ (2000) guidelines (Warne, 2001). All results seem to indicate that LRA is sufficiently protective and the most accurate of the acute to chronic extrapolation techniques when using the extrapolated LC_{50} /5 method. The extrapolation methods were validated here using very limited experimental chronic toxicity data. Further research is needed to validate the extrapolation methods for more toxicants and using more test organisms.

4.4.2 The value of extrapolated chronic toxicity data in guideline development

ACRs have been utilised in the generation of the South African Water Quality Guidelines (DWAF, 1996) (Roux *et al*., 1996) and the ANZECC and ARMCANZ (2000) trigger values (Warne, 2001). The ACR method as an acute to chronic extrapolation technique has been criticized. The numerical value of any particular ACR is dependant on the biological responses measured in respective acute and chronic toxicity tests (Warne, 1998) and, unlike the LRA method, does not take exposure time to toxicants into account. The accuracy of generic ACRs, such as the value of 10 used in the AF method to derive the ANZECC and ARMCANZ (2000) trigger values, are doubtful since tolerance of any particular species varies greatly according to the toxicant it is exposed to (Warne, 1998). The greatest shortcoming of

ACRs is however that they have little theoretical basis for their use, unlike the LRA method which has sound theoretical principles and who's accuracy and protectiveness has been validated (Mayer *et al*., 1994; Chapter 4). ACRs as an acute to chronic extrapolation method have been found to be less protective and accurate than LRA and MPA (Chapter 2 and 4).

Within the various water quality guidelines that exist, chronic toxicity data have varying degrees of importance. The process used to derive the South African Water Quality Guidelines (DWAF, 1996) is described in Chapter 1. For each toxicant, the South African Water Quality Guidelines (DWAF, 1996) define a Target Water Quality Range (TWQR), a Chronic Effect Value (CEV) and an Acute Effect Value (AEV). The AEV in the South African Water Quality Guidelines (DWAF, 1996) is used as a danger sign indicating urgent management attention, and was not meant for setting water quality requirements for aquatic ecosystems. The CEV has more weight than the AEV within the South African Water Quality Guidelines (DWAF, 1996), since the CEV can be used to set water quality requirements, and does not simply act as a warning sign. The CEV was compiled using chronic toxicity data while the AEV was compiled using acute toxicity data (Roux *et al*., 1996). The importance therefore of chronic toxicity data to the South African Water Quality Guidelines (DWAF, 1996) should not be underestimated. Where there were not enough chronic toxicity data available to derive a CEV, the Final Acute Value (one of the steps to derive the AEV: see Chapter 1) was subjected to an Acute to Chronic Ratio (ACR) and unlike the ANZECC and ARMCANZ (2000) trigger values, the South African Water Quality Guidelines (DWAF, 1996) do not group guidelines according to confidence, with CEVs derived using ACRs carrying as much weight as CEVs derived using chronic toxicity data. The LRA or MPA extrapolation method could replace the use of the ACR in this context. Extrapolated chronic toxicity data for indigenous aquatic organisms can play an important role in updating the present guidelines, since the South African Water Quality Guidelines (DWAF, 1996) are based on mainly international toxicity data.

Jooste and Rossouw's (2002) benchmarks were aimed at quantifying the water quality ecological Reserve required under the National Water Act (no 36 of 1998) and were driven by the need for numerical values defining different ecological categories i.e.

Natural, Good, Fair and Poor. The process of deriving the benchmarks is described in Chapter 1. Toxicity data were collected and used to derive the benchmarks. Jooste and Rossouw (2002) realised the inadequacy of much toxicological data due to standardisation of exposure times, regardless of test organism lifecycles. They promoted the idea of an incipient or time independent measure i.e. a measure of toxicity at infinite exposure. This is consistent with the theory behind LRA (Chapter 2, Fig 2.2). The Jooste and Rossouw (2002) benchmark method attempted to extrapolate toxicity data to an expected toxicity after 336 hours. In this way, they tried to obtain a threshold LC_{50} in much the same way the theory of LRA advocates. Their (Jooste and Rossouw, 2002) observation that LC_{50} values in investigated toxicity tests change little after 10 days was also observed for short – term chronic data in this investigation (Chapter 2 Fig 2.19 and 2.20). The ANZECC and ARMCANZ (2000) trigger values regarded all toxicity tests for multi-cellular organisms with an exposure time of more than 96 hours as chronic tests (Warne, 2001). Jooste and Rossouw (2002) however have distinguished between acute and chronic tests in terms of lethal and sub-lethal biological measures, with acute exposure time being standardised at 14 days. The sub-lethal effects benchmark, derived using toxicity data with sub-lethal biological response measures, marks the boundary between the Good and Natural health classes while the lethality benchmark marks the upper boundary between the Poor and Fair classes. Since most acute data measure lethality while most chronic toxicity data measure sub-lethal biological responses (Rand, 1995), chronic toxicity data are important for accurately defining the ideal natural water quality condition of water resources. Toxicity data used to derive the generic benchmarks, similar to the South African Water Quality Guidelines (DWAF, 1996), were collected predominantly from foreign sources. Extrapolated chronic toxicity data for indigenous organisms can play an important role in modifying the generic boundary values to be more representative of South African aquatic biota.

Scherman *et al*. (2003) attempted to link ecotoxicology, water chemistry and biomonitoring (see Chapter 1) and utilized low effect LC_x values from regression analysis of acute and short-term chronic toxicity tests to define the boundaries between ecological health classes. These boundaries were adjusted according to water chemistry and biomonitoring data. For instance, where biomonitoring results indicated a higher management class than the water chemistry results would indicate

according to toxicity tests, the class boundaries were adjusted. This is a practical and purely site - specific method of setting management classes, using reasonably easy to implement acute and short – term chronic tests. There are possibly a few problems with the method. The method utilises lower effect levels from the toxicity tests, such as LC_1s and LC_5s. These effect levels have low confidence and are model dependant with different models potentially producing drastically different values for these effect levels (Moore and Caux, 1997; Clarke *et al*., 2002). Short – term chronic test data were used, which would be classified as chronic data according to the definitions used in the ANZECC and ARMCANZ (2000) trigger values (Warne, 2001). Using three replicates of these tests would have provided the opportunity to derive chronic NOEC values, thereby overcoming the problems with regression low effects data. Additionally, the use of acute data, even at low effect levels, may not protect the aquatic biota from long-term effects. Scherman *et al*. (2003) tried to overcome this problem by adjusting boundary values according to biomonitoring data. This does not however necessarily negate the problems of using acute toxicity data. For example, biomonitoring data may indicate that an organism is found at a certain salinity concentration, however more sensitive life stages of that organism may be inhibited by that salinity level (Kefford *et al*., 2004b). A distribution of species sensitivity is not attempted, and results of toxicity tests on a few laboratory suited (and therefore probably robust) test organisms are assumed to be indicative of the sensitivity of the entire aquatic community at a certain site. Despite the aforementioned shortcomings, this method subscribes to the Precautionary Principle as defined by Warne (1998), in terms of providing a method in response to a need for urgent regulatory action, despite the fact that scientific certainty is lacking. This method could be modified by applying LRA or MPA to the acute and short-term chronic toxicity test data generated, thereby providing an estimate of chronic time scale tolerance of the organisms tested.

The process of deriving the ANZECC and ARMCANZ (2000) trigger values is described in Chapter 1. Chronic and multispecies toxicity data were used to derive high reliability (HR) trigger values while acute toxicity data were used to derive the medium reliability (MR) and low reliability (LR) trigger values (Warne, 2001). In fact, for the MR and LR trigger values, chronic response was extrapolated from acute toxicity data using ACRs (Warne, 2001). This highlights the importance of chronic

toxicity data to the trigger values. The process classified toxicity data according to exposure time (Warne, 2001), unlike Jooste and Rossouw's (2002) method of distinguishing between lethal and sub-lethal biological response data. Chronic toxicity tests for multicellular organisms were defined as toxicity tests where exposure time exceeded 96 hours while acute toxicity tests were defined as tests with an exposure time between 24 and 96 hours duration (Warne, 2001). Jooste and Rossouw (2002) standardised acute exposures to 14 days, as this is when LC_{50}s are likely to have stabilised within tests. In this way, acute and chronic toxicity data derived by Jooste and Rossouw (2002) are probably much more protective, and this is a possible serious shortcoming of the ANZECC and ARMCANZ (2000) trigger values. If insufficient chronic tolerance data were available to derive HR trigger values, MR trigger values were attempted using predicted chronic data derived by the application of Acute to Chronic Ratios to acute data (Warne, 2001). The process used to derive the ANZECC and ARMCANZ (2000) trigger values could be refined by replacing the use of ACRs with the LRA or MPA method when deriving MR TVs.

The results of this study show that the LRA $LC_{50/5}$ acute to chronic extrapolation method is the most accurate when comparing extrapolated chronic toxicity data to experimentally derived chronic toxicity data. The review of guideline development methods (Chapter 1) suggest that at present the Species Sensitivity Distribution method used in the ANZECC and ARMCANZ (2000) trigger values is the most progressive. The SSD method is a fairly new method and must be proven to be environmentally realistic, therefore some caution should be shown in using it uncritically. Future South African freshwater quality guidelines for toxicants can be refined by utilising experimentally derived chronic toxicity data for indigenous aquatic organisms and extrapolated chronic toxicity data using the LRA $LC_{50}/5$ acute to chronic extrapolation method where the acute data are for indigenous aquatic biota. Applying Species Sensitivity Distributions to these data and relating degrees of protection offered by the resulting PC_x values to boundaries of the Excellent, Good, Fair and Poor classes will refine existing water quality guidelines and boundary values.

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